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Synthesis and characterization of new bonded stationary phases for HPLC

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NOTE TO USERS

SYNTHESIS AND CHARACTERIZATION OF NEW BONDED STATIONARY PHASES FOR HPLC

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by

Deepika Janga

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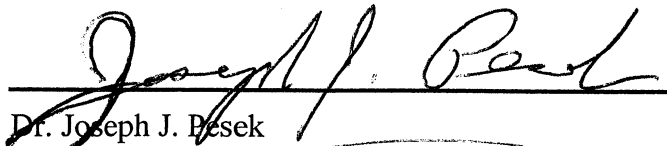
APPROVED FOR THE DEPARTMENT OF CHEMISTRY



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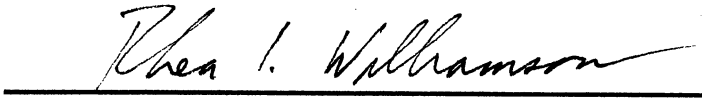

ABSTRACT

SYNTHESIS AND CHARACTERIZATION OF

NEW BONDED STATIONARY PHASES FOR HPLC

by Deepika Janga

The preparation and chromatographic evaluation of newly synthesized silica-based stationary phases for high performance liquid chromatography (HPLC) are described. The silica modification procedure follows a previously established method consisting of catalytic and free-radical hydrosilation of olefins and alkynes on a hydride modified intermediate to obtain new bonded phase packings. Columns are spectroscopically characterized using DRIFT, ^{13}C CP-MAS NMR and the quantitative information of the bonded organic moiety on the hydride surface is provided by elemental analysis. Preliminary chromatographic evaluation of the newly synthesized columns is done using selected probes including tocopherols as well as selected polyclic aromatic hydrocarbons and steroids operating under both normal and reversed-phase conditions. Further chromatographic evaluation of these columns can be done using different test solutes and also operating under different mobile phase conditions.

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Deepika Janga

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. INTRODUCTION

1.1 Background

Synthesis of stationary phases for use in High Performance Liquid Chromatography (HPLC) by chemically bonding a desired organic moiety to the silica hydride surface and characterizing these bonded phases both spectroscopically and chromatographically is the main focus of this work. Phases made by covalently bonding a molecule onto a stationary phase results in the surface coatings which are permanent. Silica, a chemically reactive substrate, was used as the support for the bonded phase packings to which various organic functionalities were bonded. Silica has wide spread utility as it is available in numerous physical forms and it is possible to control properties like surface area and pore size quite precisely. The preparation of novel anthracene-based bonded phases, 9-vinyl anthracene and 9,10-bis(phenylethynyl)anthracene using the silanization/hydrosilation approach has been part of the focus in this research work. Spectroscopic methods including diffuse reflectance infra-red fourier Transform Spectroscopy (DRIFT) and ^{13}C cross polarisation magic angle spinning spectroscopy (CP-MAS) and elemental analysis were utilized to characterize these modified surfaces in order to better understand their role in various analytical applications. Anthracene-based stationary phases on the silica support can serve as a new search tool for characterizing polycyclic aromatic hydrocarbons already known and also of the newer ones continually being discovered. Also, these bonded phases have the potential uses of separating biological molecules such as vitamins and steroids. Similarly, focus was made on but not

limited to the synthesis and characterization of ethisterone bonded phase, a novel organic moiety containing a steroid skeleton and showing some special selectivity towards steroids.

The simplest HPLC system is made up of a solvent pump, an injector, a column, a detector and a data recorder as shown in Figure 1 [1]. Since the stationary phase is composed of micron size porous particles, a high pressure pump is required to move the mobile phase through the column. The chromatographic process begins by injecting the solute onto the top of the column packed with a solid separating material also referred to as the stationary phase. Separation of the components occurs as the analytes and the mobile phases are pumped through the column. Eventually, each component elutes from the column as a narrow band or a peak on the recorder. The response of the detector to each component is displayed on a chart recorder or computer screen as a chromatogram. To collect, store and, analyze data, computers and integrators are used. Since the real work in an HPLC system occurs in the column, it is called the "heart" of the system.

Silica gel, which is the basic support of most column packings, is a hydrated silicic acid with a controlled amount of water of hydration. Each silicon atom on the surface of the packing has one or more hydroxyl groups associated with the water of hydration. The available proton on the hydroxyl group gives silica its acidic nature and along with the hydration shell makes it a very polar surface [2]. The main purpose of surface modification is to shield these polar silanol groups and to transform the surface into the hydrophobic one. Organosilanization, a surface modification procedure, is

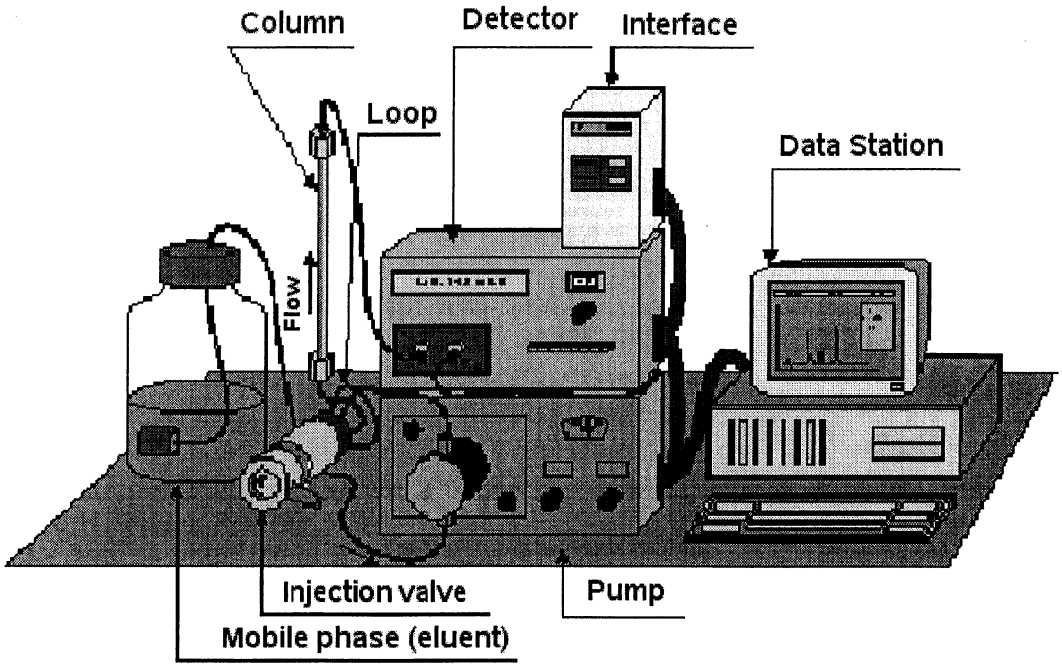


Figure 1. Functional schematic of a modern HPLC instrument.

considered a well-established synthetic technique for the preparation of HPLC packings. The procedure was derived from "silane-coupling" methodology which has been extensively utilized for making surface composites [3]. In silane coupling chemistry, organosilanization of a metal oxide substrate is carried out by reacting an organosilane of the formula $RSiX_3$ with the inorganic surface:

$$|MOH + RSiX_3 \rightarrow |M\text{-}O\text{-}Si\text{-}R + 3HX \qquad (1)$$

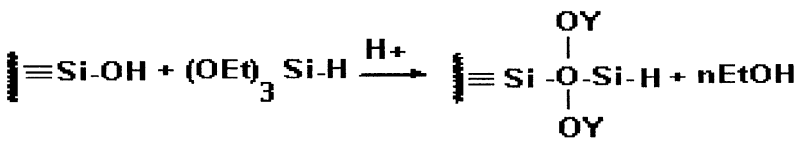
Typically, porous silicas are reacted with organosilanes to yield an Si-R functionality attached to the support through an Si-O-Si (siloxane) linkage.

$$|Si\text{-}OH + R_{4-n}SiX_n \rightarrow |Si\text{-}O\text{-}SiX_{n-1}R_{4-n} + HX \qquad (2)$$

where n = 1-3, R is an alkyl or substituted alkyl group, X is an easily hydrolyzable group such as halide, amine, alkoxy or acyloxy, and the vertical line represents the support surface. When di- or trifunctional silanes (n= 2,3) are repeated with silica in the presence of a known amount of water, the result is the formation of "polymeric" bonded phases where the thickness of the organic layer depends on the reaction conditions. Traditionally, these bonded phases have been considered disadvantageous with respect to their monomeric (n=1) counterparts, presumably because of the difficulties associated with the control of the polymerization process which frequently results in irreproducible

phase thickness and limited column efficiency [4]. It has been found that the size of the organic group 'R' strongly determines the extent at which siloxane linkages (M- O- Si) are formed. The "bulkier" the group is, the smaller the extent of condensation and, hence, the greater the tendency to contribute additional silanols to the bonded phase [5]. These unreacted silanol groups then impart an undesirable polarity to the surface which may lead to the tailing of the chromatographic peaks. To lessen this effect, end-capping is used, the process which involves the use of small organosilane reagents such as trimethylchlorosilane (TMCS) and hexadiethylsilizane (HMDS) for removing many of the unreacted and accessible silanol groups on silica as well as for increasing the hydrophobicity of the surface [6,7,8]. Since not all of the silanols are removed, this approach does not provide a total solution to the problem.

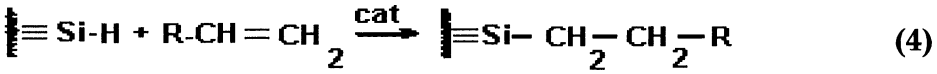
More than a decade ago, the silanization/hydrosilation process was developed to overcome the drawbacks associated with the use of organosilane reagents for the synthesis of HPLC stationary phases [4,5]. In the process of silanization, the bulky organic group 'R' that contributed additional silanols to the bonded phase was replaced by hydrogen such that the reaction proceeded with a maximum of siloxane formation. In this work, the hydride-modified support was formed by reaction of triethoxy silane (TES) with a silica substrate in the presence of water, an acid catalyst, and an appropriate solvent. The process can be described as follows :



(3)

where Y = Si or H depending on the extent of crosslinking.

The new hydride intermediate is stable in the presence of air and water, and also, it can be stored for long periods of time without appreciable decomposition. The hydrosilation step involves attachment of an appropriate organic moiety to the hydride surface. This reaction leads to the formation of a direct Si-C bond at the surface that has been shown to be more hydrolytically stable than the Si-O-Si-C linkage formed during an organosilanization reaction [9,10].



(4)

A terminal olefin is generally used for bonding to the Si-H group, however, other types of unsaturated compounds such as acetylenes, nitriles and isocyanates have also been used [11]. The reaction is facilitated by the presence of the catalyst such as a transition metal complex (Speier`s catalyst- 2-propanol solution of hexachloroplatinic acid). Another approach for attaching the organic moiety to the hydride was via free radical addition using an initiator such as t-butyl peroxide. The final product of hydrosilation results in a bonded material that is similar to the monomeric products from organosilanization. The major difference being that the organosilanization material has silanol groups remaining on the surface while the silanization/hydrosilation product has predominantly hydrides below the bonded organic moieties. The process of silanization / hydrosilation seems quite well suited for the preparation of anthracene-based and steroid-

based bonded phases as the compounds under study also possesses terminal alkene and alkyne functional groups whose structures are shown in Figure 2.

In order to elucidate the exact nature and structure of a particular bonded moiety, both DRIFT and ^{13}C CP-MAS NMR spectroscopies were used. Also,elemental analysis was used to determine whether an organic moiety was present as well as to provide some quantitative information based on the known structure of the compound. This assessment was simply done by carbon analysis since the oxides used in these applications do not have this element present under normal circumstances.

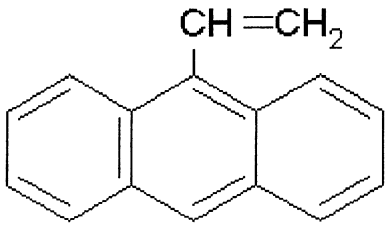
Bonded phase packings thus synthesized by silanization/hydrosilation approach were further characterized chromatographically by doing some preliminary tests under both normal phase and reversed phase chromatographic conditions. This proves its advantages over bare silica which is a classic normal phase material. They are also faster to equilibrate, use less organic solvents and offers more versatile surface chemistries than bare silica. The separation is accomplished based on the differences in the compound polarity with respect to the relative polarities of the mobile phase and stationary phase. The HPLC system is referred to be in normal phase mode when the stationary phase is highly polar and the mobile phase is relatively non-polar with respect to the polarity of the solutes. In this phase, the least polar solutes elutes first because they are the most soluble in the mobile phase and increasing the polarity of the mobile phase decreases the elution time. In reversed-phase mode, the stationary phase is non-polar and the mobile phase is relatively polar with respect to the polarity of the solutes. The separation of the

solutes occurs with the most polar components appearing first and increasing the polarity of the mobile phase increases the elution time [12].

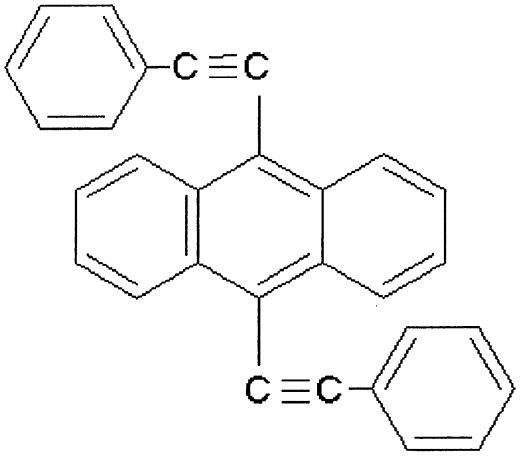
In this work, separations on anthracene and steroid based columns were done isocratically using 4 different compositions of highly polar mobile phases such as mixtures of methanol-water, and acetonitrile-water respectively when the system is in the reversed-phase mode. And, in the normal phase mode, a highly non-polar solvent such as hexane was mixed in 4 different compositions with various other solvents such as chloroform, methylene chloride and amyl alcohol. In this manner, the polarity of the stationary phase was matched roughly with that of the analytes and then a mobile phase of considerably different polarity was used for elution.

1.2 Aims of this Work

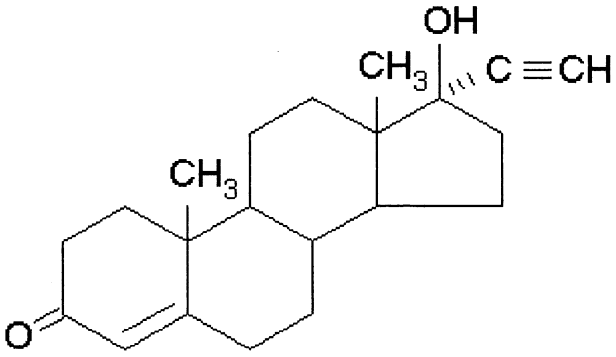
One major goal of the present research is to prepare anthracene-based and steroid-based bonded phases by applying both catalytic and free radical hydrosilation as the main surface bonding reaction. IR and NMR spectrum characterizations of the resulting bonded materials are also important aspects of this work. Finally, the chromatographic characterization of the bonded phases using selected PAHs, vitamins and steroids under normal phase and reversed phase chromatographic conditions is another major goal of the present research. A comparison of the separation performance of the new bonded phase materials is also an important part of the present work.



9-Vinylanthracene



9,10-Bis(phenylethynyl)anthracene



Ethisterone

Figure 2. Structures of the organic compounds used for the bonded phase synthesis.

2. EXPERIMENTAL

2.1 Materials and Reagents

All the stationary phases were prepared using Kromasil® 100 (AKZO NOBEL, Bohus, Sweden) silica gel having a particle diameter of 5 µm, a pore diameter of 100° A and a surface area of 340 m^2/g. For chemical modification of the silica surface, 2.3 M HCl (Fisher Scientific) and Triethoxy silane (Sigma-Aldrich, Milwaukee,WI) were used. The organic compounds 9-vinyl anthracene, 9,10- bis(phenylethynyl)anthracene and ethisterone were all purchased from Aldrich Chemical Co. (Milwaukee, WI). A 10 mM hexachloroplatinic acid solution in 2-propanol and t-butyl peroxide were both purchased from Aldrich Chemical Co. (Milwaukee, WI) and used as catalysts for hydrosilation. The solvents used in the synthetic reactions, ethanol, p-dioxane and diethyl ether were all obtained from Fisher Scientific and were reagent grade. LC grade hexane, chloroform, methylene chloride, and acetonitrile were the mobile phase solvents used and were obtained from Aldrich Chemical Co. (Milwaukee, WI). Deionized water was prepared on a Milli-Q™ purification system (Millipore Corp., Bedford, MA). Polycyclic aromatic hydrocarbons, naphthalene, anthracene, acenaphthene and fluorene, were used as test solutes and were obtained from the National Institute of Standards and Technology (Gaithersburg, MD). Vitamins, d-α tocopherol, d-δ tocopherol and dl- α tocopherol were another group of test solutes used and were obtained from ICN Biomedicals Inc. (Irvine,CA). Steroids, adrenosterone, corticosterone, estradiol, prednisone, estrone, 11-α acetoxy progesterone, were all obtained from Sigma- Aldrich (Saint Louis, Missouri).

2.2 Instrumentation

2.2.1 Infrared Spectrometry

Fourier-transform spectra for 9-vinyl anthracene, 9,10 bis(phenylethynyl) anthracene and ethisterone bonded phase samples were obtained from AT1 Mattson Infiniti series-FTIR . The FTIR spectrometer equipped with a deuterated triglycine sulfate (DTGS) detector was used for Diffuse Reflectance Infrared Fourier Transform (DRIFT) spectra. A DRIFT accessory equipped with a sample cup was used for analysis. The bonded phase sample was mixed with 5% (by weight) of KBr and the mixture was finely ground and filled to the sample cup with a smooth sample surface. An IR spectrum of light reflected from the surface will show absorption peaks characteristic of the molecule and its method of bonding to the surface. Spectra were collected in the mid-infrared region of 4000-450 cm^{-1} with 100 sample scans ratioed against pure KBr as reference and the spectra were normalized to 100% transmittance.

2.2.2 Nuclear Magnetic Resonance

Proton and carbon NMR spectrum were obtained on a Radian NMR Model 400 for identification of the organic compounds under study. The NMR spectrum measurements were performed using 1mg of sample dissolved in 750µl deuterated chloroform ($CDCl_3$) solvent in a 5mm o.d glass tube (Wilmad Glass Co.,Inc.). Proton chemical shifts were referenced to $CDCl_3$ which gave the peak at 7.2 ppm from TMS. Carbon-13 shifts were referenced to solvent $CDCl_3$ peaks at 76.8, 77.2 and 77.4 ppm

from TMS.

Solid state NMR spectrum spectra with cross polarization and magic angle spinning(MAS) were obtained on a 300-MHz General Electric Model QE 300 spectrometer. MAS was obtained at a spinning rate of 5 KHz and 300 to 500 mg of bonded silica sample was measured in a double bearing ZrO_2 rotor.

2.2.3 Elemental Analysis

Carbon analysis was carried out by Desert Analytics (Phoenix, Arizona). For each analysis, 5mg of the sample was sent and the results were obtained as the percentage of the carbon element in the bonded organic molecule.

2.2.4 Column Packings

Columns were packed by the slurry method using a CCl_4 - methanol (9:1 v/v) mixture. The bonded material was packed into a 10cm×0.46cm i.d. stainless-steel tube (Alltech, Deefield, IL) using a pneumatic pump operated at 5000psi pressure with methanol as the driving solvent.

2.2.5 Liquid Chromatography

HPLC measurements were made with a liquid chromatograph system equipped with a Waters 515 quaternary gradient pump (serial # G97915 793M), a manual injector, a variable wavelength photodiode array detector (Waters 991) and an integrator when the system is operating in the normal-phase mode. In the reverse phase mode, a Hitchai liquid Chromatograph system connected to a PC-based data system and equipped with

Millenium software was used for HPLC measurements.

2.3 Procedures

2.3.1 Preparation of Bonded Phases

2.3.1.1 Silanization with TES

Prior to the synthesis, 5.00-g of silica was weighed and dried overnight at 100°C in a vacuum oven. While drying, care was taken to avoid spilling in the oven by covering the beaker of silica with a watch glass. In a typical procedure, 5.00-g of dried silica was placed in a 3-neck, 500 ml round bottom (RB) flask equipped with a thermometer, a condensor with a drying tube, an addition funnel with an equalizing tube, a heating mantle and a magnetic stirrer. Then, 200-ml of reagent grade dioxane was added followed by 2.43 ml of (aqueous) 2.3M HCl. The mixture was heated to about 70°C, and then 30.05-ml of dioxane was transferred into the addition funnel equipped with a stopper. Later, under argon, 6.95-ml of TES was added slowly to the addition funnel containing dioxane. The TES/dioxane mixture was then added dropwise into the RB flask over a period of 15-20 min with constant stirring. The temperature was raised to 90° C and the mixture was allowed to reflux for 90 min. After a cooling period, the product was carefully transferred into the centrifuge tubes with stir bars and was centrifuged at 1500 rpm for 10 min. Later, using vacuum suction, the upper layer containing the TES/dioxane mixture was filtered leaving the solid silica hydride layer. Then a 50-ml portion of dioxane was added to the silica hydride layer and stirred for 30 min on a stir

plate. Later, it was centrifuged for 10min at 1500 rpm. The process was repeated twice with 100-ml portions of dioxane, 3 times with 150-ml portions of toluene and 3 times with 150-ml portions of diethyl ether. The final product was dried at room temperature overnight to evaporate the ether and finally dried in a vacuum oven at 110°C for 6 h or more [5].

2.3.1.2 Hydrosilation using Speier's Catalyst

2.3.1.2.1 BPEA Bonded Phase

Prior to hydrosilation, all the glassware used was dried in the oven and Kromasil silica hydride was dried overnight in a vacuum oven at 110°C. 6.56-g of 9,10-bis (phenylethynyl) anthracene (BPEA) was dissolved in 57 ml of chloroform and was transferred into a 250-ml round bottom flask. Next, 5.3 ml of 10mM hexachloroplatinic acid in 2-propanol (Speier's catalyst) was added. This mixture was then heated at 60°C for one hour while being stirred. After this " induction period ", when a clear solution was obtained, 2.0-g of hydride silica was added slowly (approximately 15-20 min) to the anthracene/catalyst solution. The reaction was allowed to proceed for 96 hours at 60°C with continuous stirring. After cooling, the bonded silica product was centrifuged and washed with four 30-ml portions chloroform, followed by similar washings with dichloromethane and diethyl ether. The final ether solvent was allowed to evaporate at room temperature overnight and the product was then dried under vacuum at 110°C for 6 hours [13].

2.3.1.2.2 Ethisterone Bonded Phase

A similar procedure as described above was used for ethisterone bonded phase synthesis. Here 6.8 g of ethisterone was dissolved in 71.45-ml of a 1:1 ratio chloroform and absolute ethanol. Next, 6.7ml of 10mM Speier's catalyst was added. After a 1 hour induction period at 60°C, 2.5 g of silica hydride was added slowly to the steroid /catalyst solution. The reaction was allowed to proceed for 96 hrs at 60°C with continuous stirring. After cooling, the washings were done with four 30-ml portions of 1:1 ratio chloroform and ethanol, followed by similar washings with dichloromethane and diethyl ether. According to Aldrich tested solubility, ethisterone was soluble in a 1:1 ratio chloroform and methanol. However, the solubility was tested using 1:1 ratio chloroform and ethanol, as methanol has a tendency to compete with ethisterone for the binding sites on silica hydride.

2.3.1.3 Hydosilation using Free-radical Catalyst

2.3.1.3.1 9-vinylanthracene Bonded Phase

Prior to hydrosilation, all the glassware used was dried in the oven and silica hydride was dried overnight in a vacuum oven at 110°C. The reaction was carried out in a 250-ml three necked round bottom flask fitted with a thermometer in one neck and a condenser equipped with a drying tube in a second neck. The third neck was closed with a glass stopper. A mixture of 35-ml of distilled toluene, 2.5-g of 9-vinylanthracene (olefin) and 0.1 ml of t-butyl peroxide (free-radical catalyst) were added to the flask at the

start of the synthesis. This mixture was heated to 60-70°C and stirred for 1 hour. Then the silica hydride was added slowly to the flask through the open neck after removal of the glass stopper. The flask was then flushed with nitrogen and all the joints were properly sealed. The flask was then closed and the temperature of the solution with the solid hydride silica was raised and maintained at 100±2°C with continuous stirring for 100 hours. After this period, the solvent was removed with vacuum suction and the solid was washed with four 30-ml portions of toluene, followed by similar washings with methylene chloride and diethyl ether. The product was first dried for several hours at room temperature and then atleast overnight at 110°C under vacuum [14].

2.3.1.3.2 Ethisterone Bonded Phase

A mixture of 70.4-ml 1:1 ratio chloroform and absolute ethanol, 7.81-g of ethisterone and 230 µl of t-butyl peroxide were added to a 500-ml round bottom flask equipped as described in the above procedure. The mixture was heated at a constant temperature of 60°C for one hour with continuous stirring. Later 2.5-g of dried silica hydride was added slowly through the open neck of the flask. The flask was flushed with nitrogen and the all the joints were sealed. The temperature of the reaction mixture was maintained at 60°C to avoid the evaporation of the solvent chloroform (boiling point of chloroform is 62°C) and the reaction was allowed to proceed for 100 hours. The final product was washed with four 30-ml portions of a 1:1 ratio chloroform and ethanol, followed by methylene chloride and diethyl ether. The product was first dried at room

temperature and later dried overnight in the vacuum oven at 110°C.

2.3.2 HPLC Separation

2.3.2.1 Tocopherols

Three tocopherols, d-α tocopherol, d-δ tocopherol and dl-α tocopherol, calciferol (vitamin D) and finally a mixture of the three tocopherols were used to test the retention behavior of the newly synthesized bonded phases. Figure 3 shows the structures of the three tocopherols and calciferol. HPLC separations were isocratically performed using the following four mobile phase compositions: 100% methanol and methanol/water in the ratios of 95:5,90:10 and 85:15 respectively in the reverse-phase mode. The flow rate was set at 1ml/min at ambient temperature with an injection volume of 20 µl. UV detection was at 293 nm. In the normal phase mode, the following mobile phase compositions were used: 100% hexane and hexane/amyl alcohol in the ratios of 95:5, 90:10 and 85:15 respectively. The flow rate was adjusted to 2ml/min and the UV detection wavelength was 293 nm.

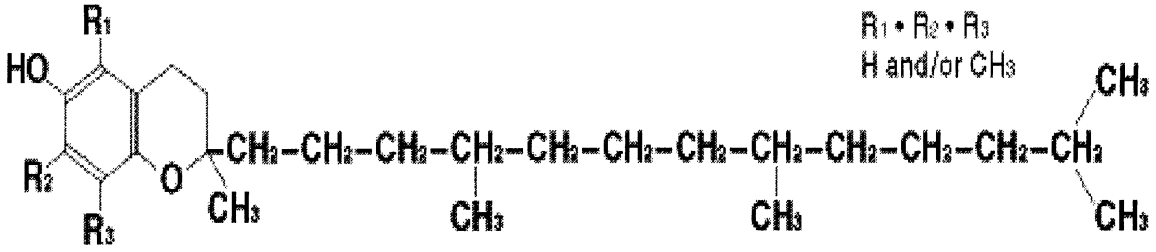
2.3.2.2 Polycyclic Aromatic Hydrocarbons

Four polycyclic aromatic hydrocarbons (PAHs), namely naphthalene, anthracene, acenaphthene and flourene, and a mixture of all four PAHs were used as test solutes to compare the retention behavior of the new bonded phases. Figure 4 shows the structures of the four PAHs used in this study. The typical sample solution was 1mg/ml with an injection volume of 20 µl and the UV detection wavelength was set at 254 nm. PAHs

were isocratically separated using 100% acetonitrile and acetonitrile/water in the ratios of 95:5, 90:10 and 85:15 respectively when operating in the reverse-phase mode. In the normal phase mode, 100% hexane and hexane/chloroform in the ratios of 95:5, 90:10 and 85:15 respectively were the mobile phases used. The flow rate was 1ml/min and the injection volume was 20 µl. The UV detection wavelength was set at 254 nm.

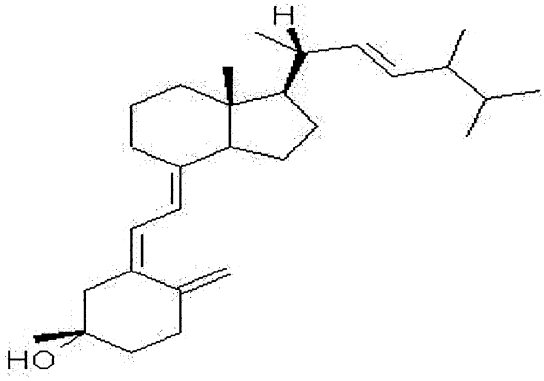
2.3.2.3 Steroids

For steroids separation, progesterone, adrenosterone, corticosterone, estradiol, prednisone, estrone, and a mixture of all the above compounds were used as the test solutes. Figure 5 shows the structures of the steroids mentioned above. HPLC separations were isocratically performed in the reverse-phase mode with four different mobile phase compositions of acetonitrile/water as described above, at 2ml/min flow rate, at ambient column temperature and, with an injection volume of 20 µl. UV detection was at 240 nm. In the normal phase mode, the following four mobile phases were used: 100% hexane and hexane/methylene chloride in the ratios of 95:5, 90:10 and 85:15 respectively. The flow rate was 2ml/in and the detection wavelength was set at 240 nm as the ketonic functional groups on the steroid molecules absorbs at that wavelength.



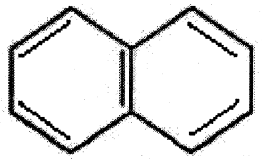
TOCOPHEROLS

d-α tocopherol	$R_1 = R_2 = R_3 = Me$
d-δ tocopherol	$R_1 = R_2 = H$, $R_3 = Me$

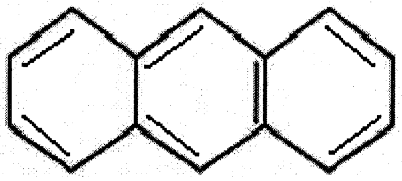


CALCIFEROL

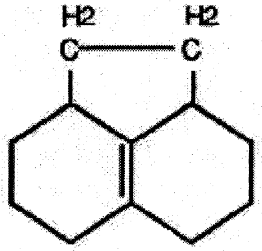
Figure 3. Structures of the vitamins used as test solutes in HPLC.



NAPHTHALENE



ANTHRACENE



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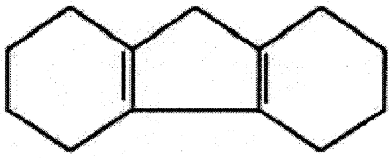
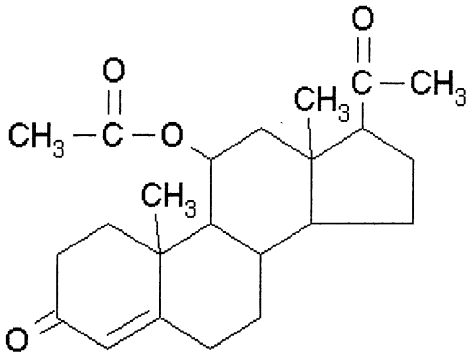
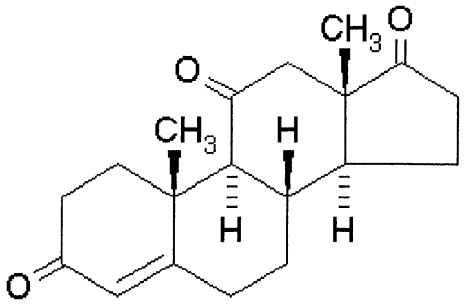


FLUORENE

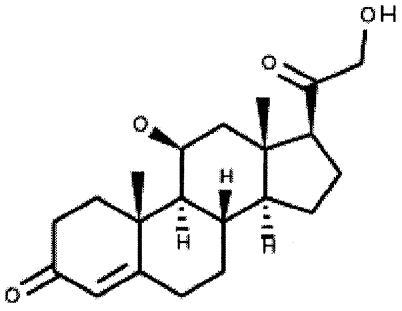
Figure 4. Chemical structures of the PAHs used as test solutes.



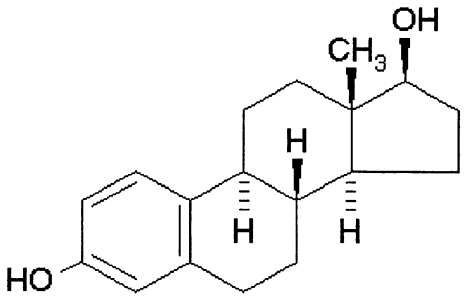
11-α-ACETOXY PROGESTERONE



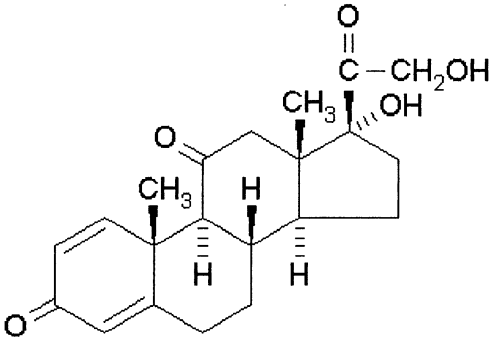
ADRENOSTERONE



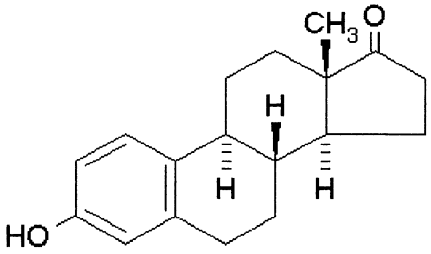
CORTICOSTERONE



ESTRADIOL



PREDNISONE



ESTRONE

Figure 5. Structures of the steroids used as test solutes in HPLC.

3. Results and Discussion

3.1 9-vinylanthracene

Proton and ^{13}C liquid NMR spectrum spectra of 9-vinylanthracene was obtained from 1mg of the sample dissolved in 750 µl deuterated chloroform. Proton chemical shifts were referenced to the $CDCl_3$ peak at 7.24 ppm from TMS. Figure 6 shows the ^{1}H NMR spectrum of 9-vinylanthracene. The distribution of protons represents a total of 12 hydrogens. The peak at 7.5 ppm (H_F) can be assigned to the proton attached to carbon atom #1 , the peak at 6.0 ppm (H_G) can be attributed to the cis proton attached to carbon atom #10, and the peak at 5.6 ppm (H_G) is due to the trans proton attached to the carbon #10 (see Figure 7). The protons (H_B and H_C) on carbon atoms 8 and 7 represents the two equal intensity multiplets at δ 7.44 ppm and, the protons (H_D) on carbon atom #5 are the multiplets at δ 7.98 ppm. A signal half as intense at δ 8.3 ppm (H_A) represents the protons on the carbon atom #9. The proton (H_E) gives the down field peak at δ 8.39 ppm (see Table1).The carbon atoms are numbered according to the decreasing order of their carbon chemical shifts. Thus, it is evident that the vinylic group is attached to the anthracene molecule.

The ^{13}C NMR spectrum of 9-vinylanthracene is shown in Figure 7. Carbon-13 shifts were referenced to the $CDCl_3$ solvent peaks at 76.8, 77.2 and 77.4 ppm from TMS. ^{13}C NMR spectrum analysis provides conclusive confirmation for the terminal $=CH_2$ structure. The downfield peak at 133.7 ppm represents the carbon atom #1 and the upfield peak at 123 ppm corresponds to carbon atom #10 which are the vinylic carbons.

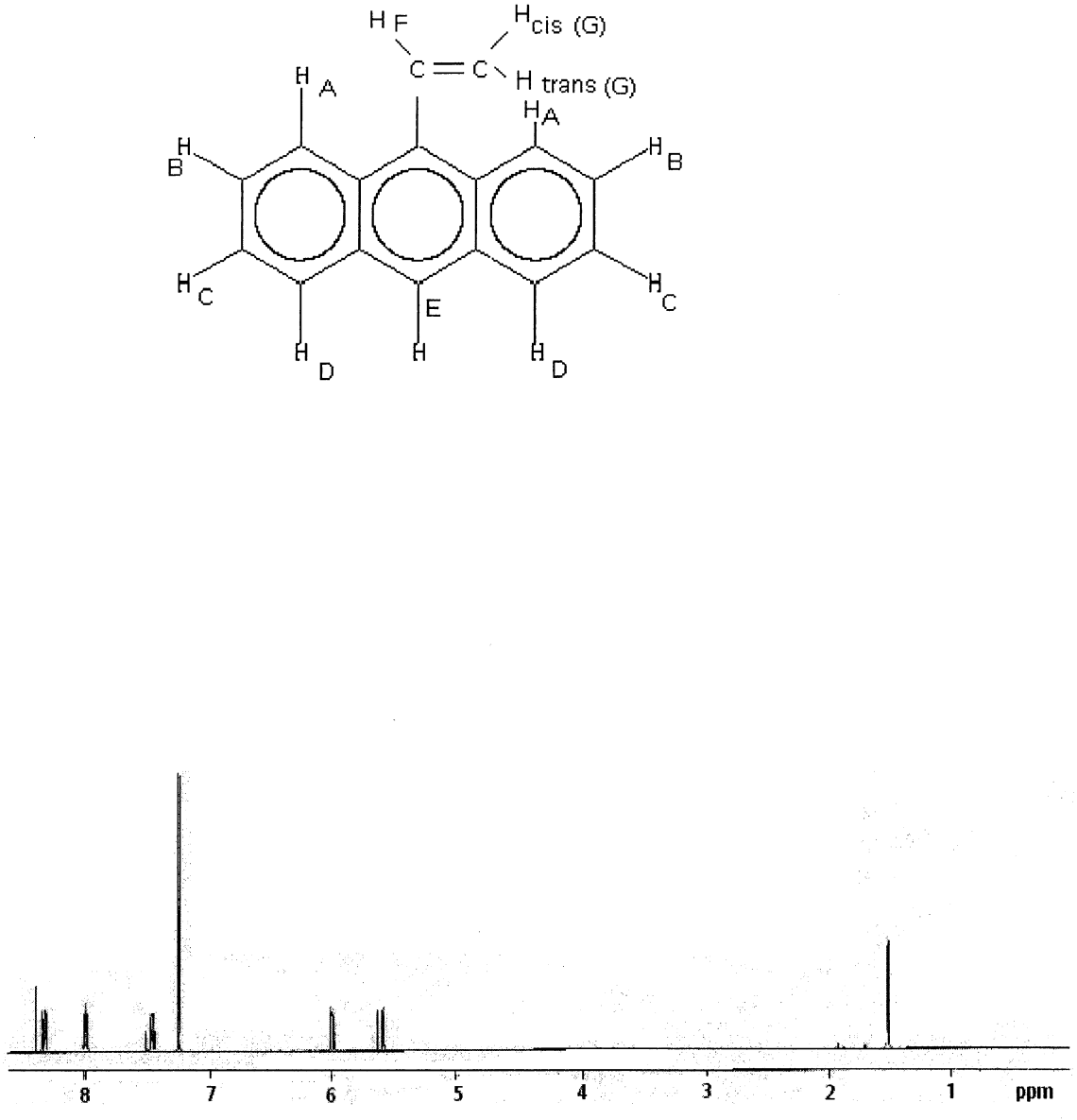


Figure 6. Proton NMR spectrum of 9-vinylanthracene.

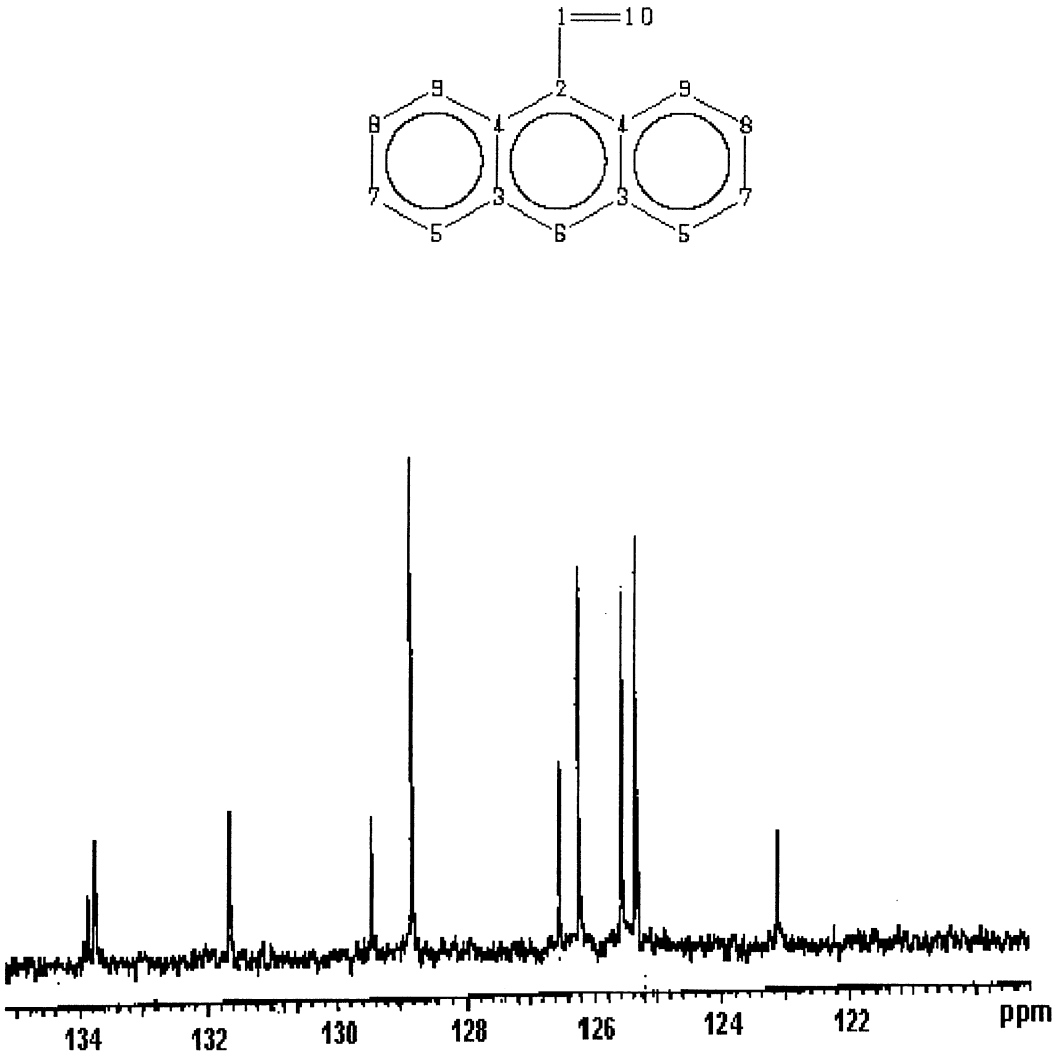


Figure 7. Carbon-13 NMR spectrum of 9-vinylanthracene.

Table 1. Proton and carbon-13 NMR chemical shifts for vinylanthracene.

Proton NMR spectrum		***Carbon NMR spectrum***	
Hydrogen	ppm	Carbon	ppm
9 ($2H_A$)	8.3	1 (1C)	133.74
8 ($2H_B$)	7.46	2 (1C)	131.3
7 ($2H_C$)	7.46	4 (2C)	129.44
5 ($2H_D$)	7.99	5 (2C)	128.8
6 ($1H_E$)	8.38	6 (1C)	126.53
1 ($1H_F$)	7.5	7 (2C)	125.89
10 (H_{Gcis}, H_{Gtrans})	6, 5.6	8 (2C)	125.56
		9 (2C)	125.31
		10 (1C)	123.1

Here, the carbon atoms are numbered according to the decreasing order of their carbon chemical shifts with reference to TMS. The chemical shifts for 9-vinylanthracene are summarized in Table1.

The bonded phase synthesis of 9-vinylanthracene was successful using free-radical catalyst, t-butyl peroxide. In the hydrosilation of this olefin using Speier's catalyst, the final product turned from pale yellow to brown. So, the above experiment was repeated by changing the reaction conditions. First, the olefin was dissolved in distilled toluene and the solution was heated for 3 days at 80°C. Following that, Speier's catalyst was added and the reaction was allowed to proceed for 2 days maintaining the temperature at 80°C. Later, when the temperature was increased to 84°C, the compound turned to brown. The olefin/catalyst mixture was allowed to dry at room temperature and later under vacuum. The proton NMR spectrum of this olefin/catalyst mixture indicated a slight upfield shift of protons attached to the anthracene molecule and also disappearance of the peaks that corresponds to the two vinylic carbons. Figure 8 shows the proton NMR spectrum of the olefin/catalyst mixture. The carbon-13 NMR spectrum of this brown product is shown in Figure 9. It showed less intense and also the disappearance of many of the carbon peaks. The brown color of the final product can be the result of the reduction of the catalyst from Pt (IV) to Pt (0) through its interaction with the hydride surface. A similar phenomenon was observed in the diol bonded phase column synthesized via hydride intermediate [15]. In this study, the authors reported that a relatively small amount of Pt on the surface can lead to the darkening of the product and

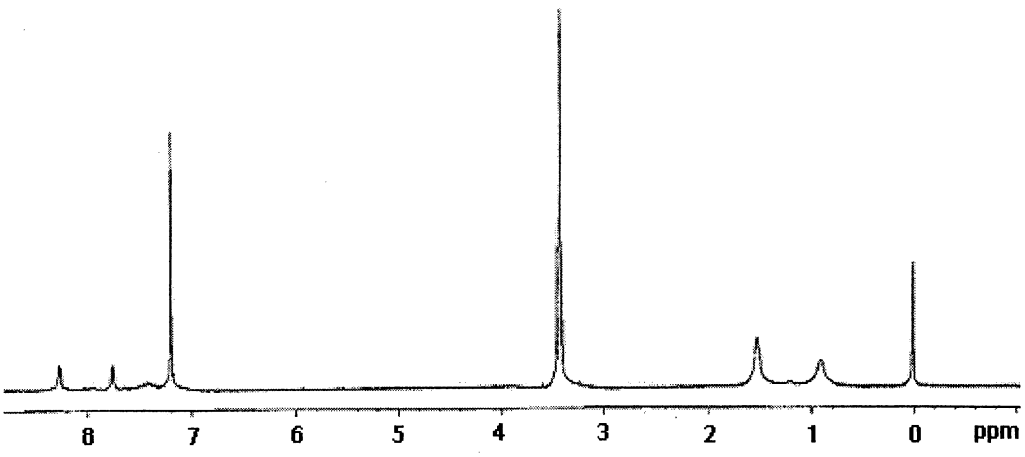


Figure 8. Proton NMR spectrum of the 9-vinylanthracene and the Speier's catalyst mixture which turned brown.

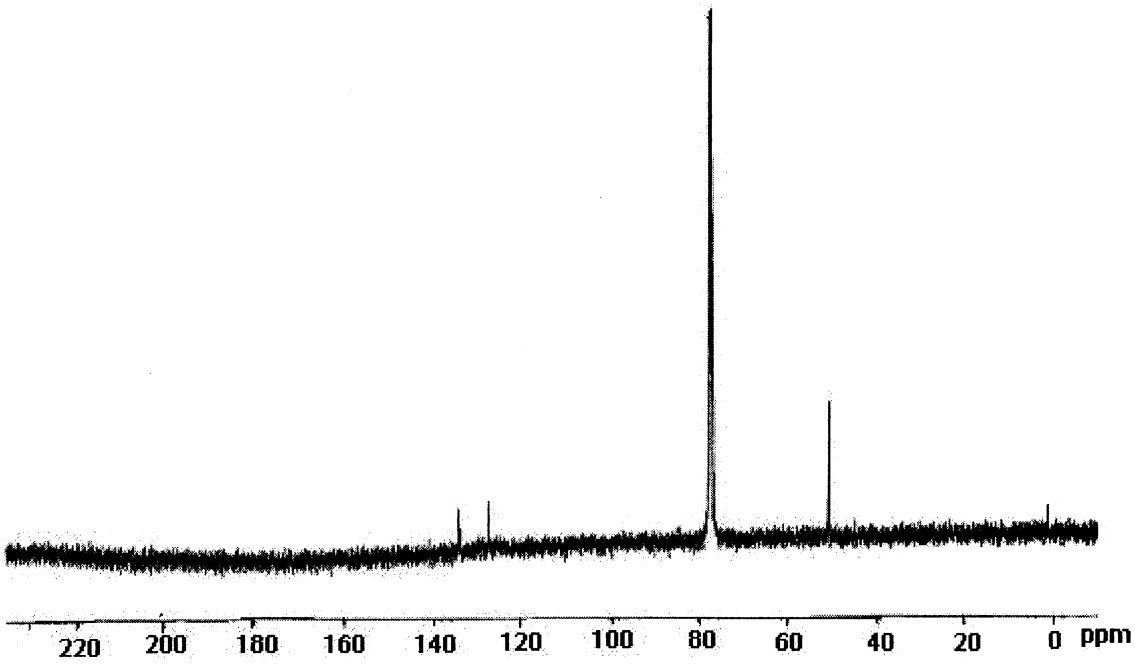


Figure 9. Carbon-13 NMR spectrum of the 9-vinylanthracene/Speier's catalyst mixture.

they also confirmed reduction of the Pt catalyst spectroscopically by high resolution ESCA spectra of the Cl 2p region. From this spectrum, it was observed that Cl on the bonded material was not coordinated to the Pt (IV) as is the case for the catalyst, but it was probably free chloride ion which was adsorbed on the surface after the platinum was reduced.

The FT-IR spectrum provides specific functional group information so that the structure of the starting material can be compared to the compound on the silica hydride surface. First, for the hydride intermediate, the success of the silanization reaction is monitored by the appearance of an Si-H stretching band at 2250 cm^{-1} [16]. Figure 10 shows the DRIFT spectrum of the Kromasil silica hydride. The broad peak between 3800 and 3000 cm^{-1} is due to adsorbed water and hydrogen bonded silanols. A peak near 3750 cm^{-1} is the OH stretch from non-hydrogen bonded silanols. The other peaks in the spectrum are due to various fundamental vibrations of the silica matrix.

After hydrosilation of 9-vinylanthracene, new infrared bands appeared which are characteristic of the organic moiety on the surface. Figure 11 shows the DRIFT spectrum of the 9-vinylanthracene bonded phase using t-butyl peroxide (free radical catalyst). The observation of strong C-H stretching vibration peaks between 3000 cm^{-1} and 2800 cm^{-1} confirms the presence of the organic moiety on the hydride surface. Also, the C=C aromatic stretching frequency at 1600 cm^{-1} confirms the presence of anthracene molecule. In addition, after the hydrosilation reaction, the Si-H peak at 2250 cm^{-1} was reduced in intensity indicating that bonding has taken place. The failure of the 9-vinylanthracene

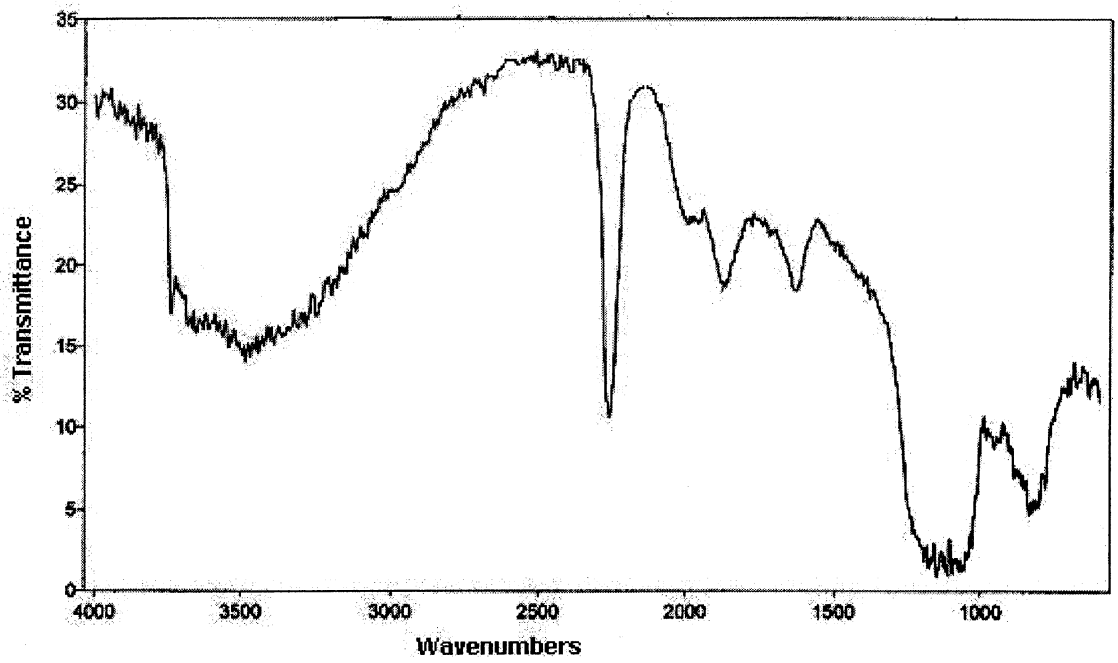


Figure 10. DRIFT spectrum of Kromasil silica hydride.

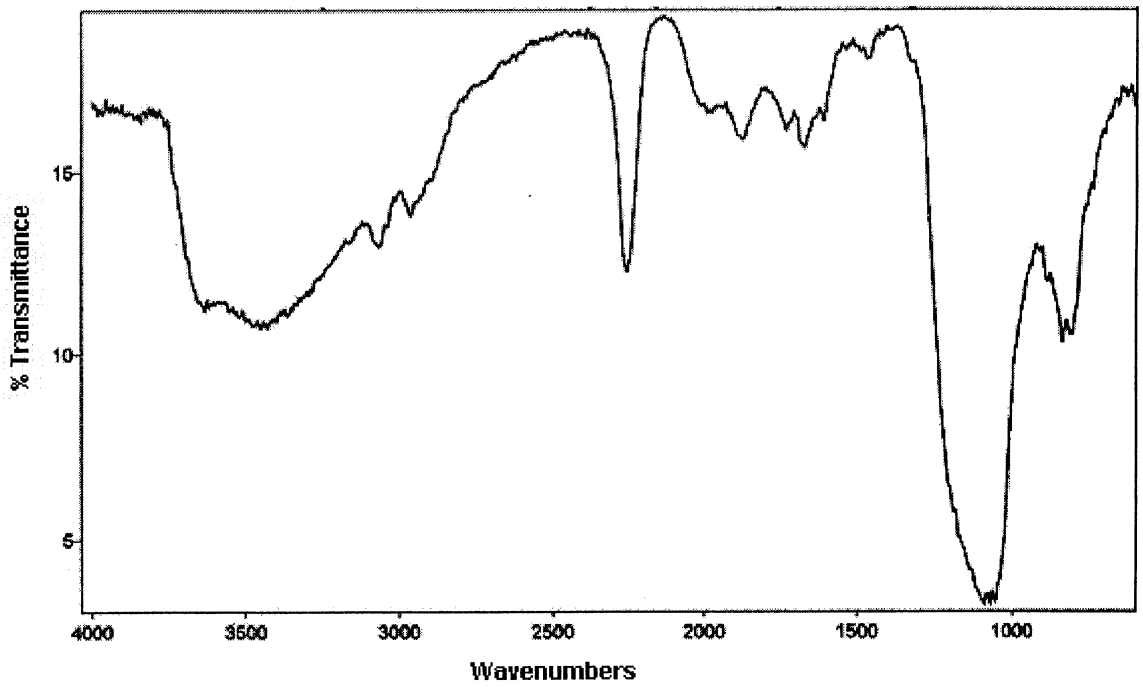


Figure 11. DRIFT spectrum of 9-vinylanthracene bonded phase using t-butyl peroxide.

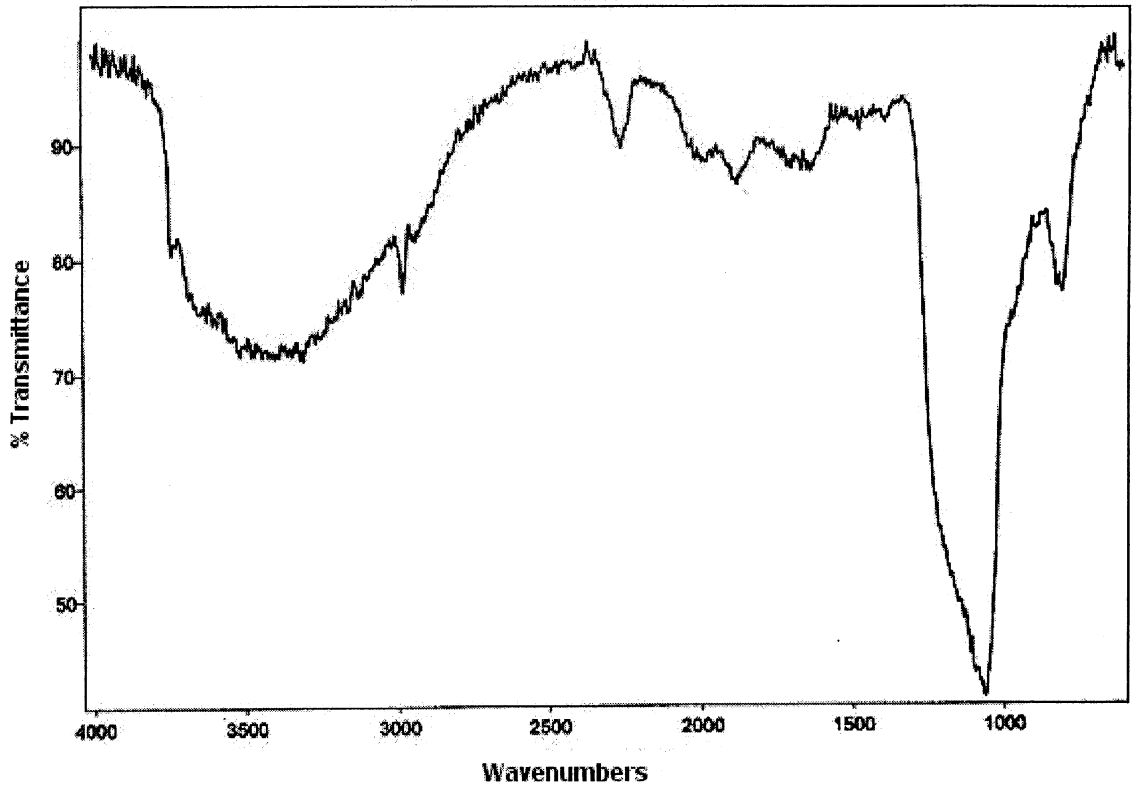


Figure 12. DRIFT spectrum of 9-vinylanthracene bonded phase using Speier's catalyst.

bonded phase synthesis using a Pt catalyst was confirmed from its DRIFT spectrum which is shown in Figure 12. The peaks due to C-H stretching vibration between 3000 and 2800 cm^{-1} and also the Si-H stretching peak at 2250 cm^{-1} were not observed which confirmed that bonding has not taken place. The carbon-13 CP-MAS NMR spectrum provides structural information about the molecule which is bonded to the silica surface. The resonances of the individual carbon atoms can be identified which confirms not only the correct structure of the bonded molecule but also confirms bonding.

Figure 13 shows the ^{13}C NMR spectrum after free radical hydrosilation reaction of 9-vinylanthracene on a hydride surface. The broad peak at 30 ppm represents both the carbon that is directly bonded to the hydride surface and the methylene carbon that is present adjacent to the bonded carbon. This confirms that the organic moiety is actually bonded to the surface as opposed to being just adsorbed. The broad nature of the peak in the CP-MAS spectrum is due to an NMR spectrum effect, most likely a significant decrease in the relaxation times of some carbons. Association of adjacent bonded moieties also results in a sharp decrease in the spin-spin relaxation time (T_2) that leads to the broadening and eventual disappearance of some resonances. The peaks at 128 ppm and 177 ppm are due to aromatic carbons of the anthracene molecule [17]. The peak at 78 ppm might correspond to a solvent that was used for washings. It is evident that there is no terminal olefin peak after bonding to silica. The terminal olefin group is involved in the direct bonding on the hydride silica in the hydrosilation reaction.

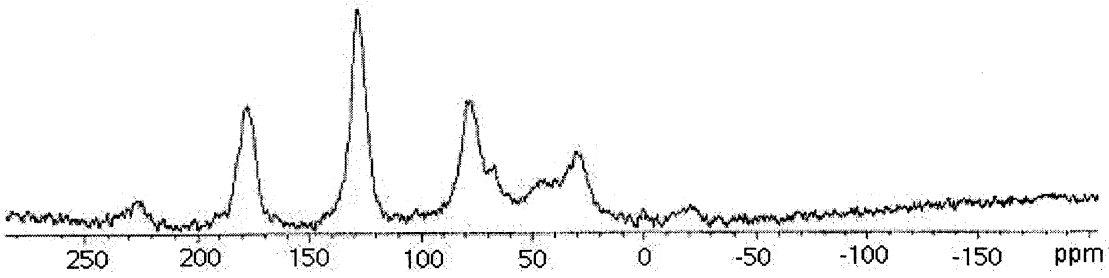


Figure 13. Carbon-13 CP-MAS NMR spectrum of 9-vinylanthracene bonded phase.

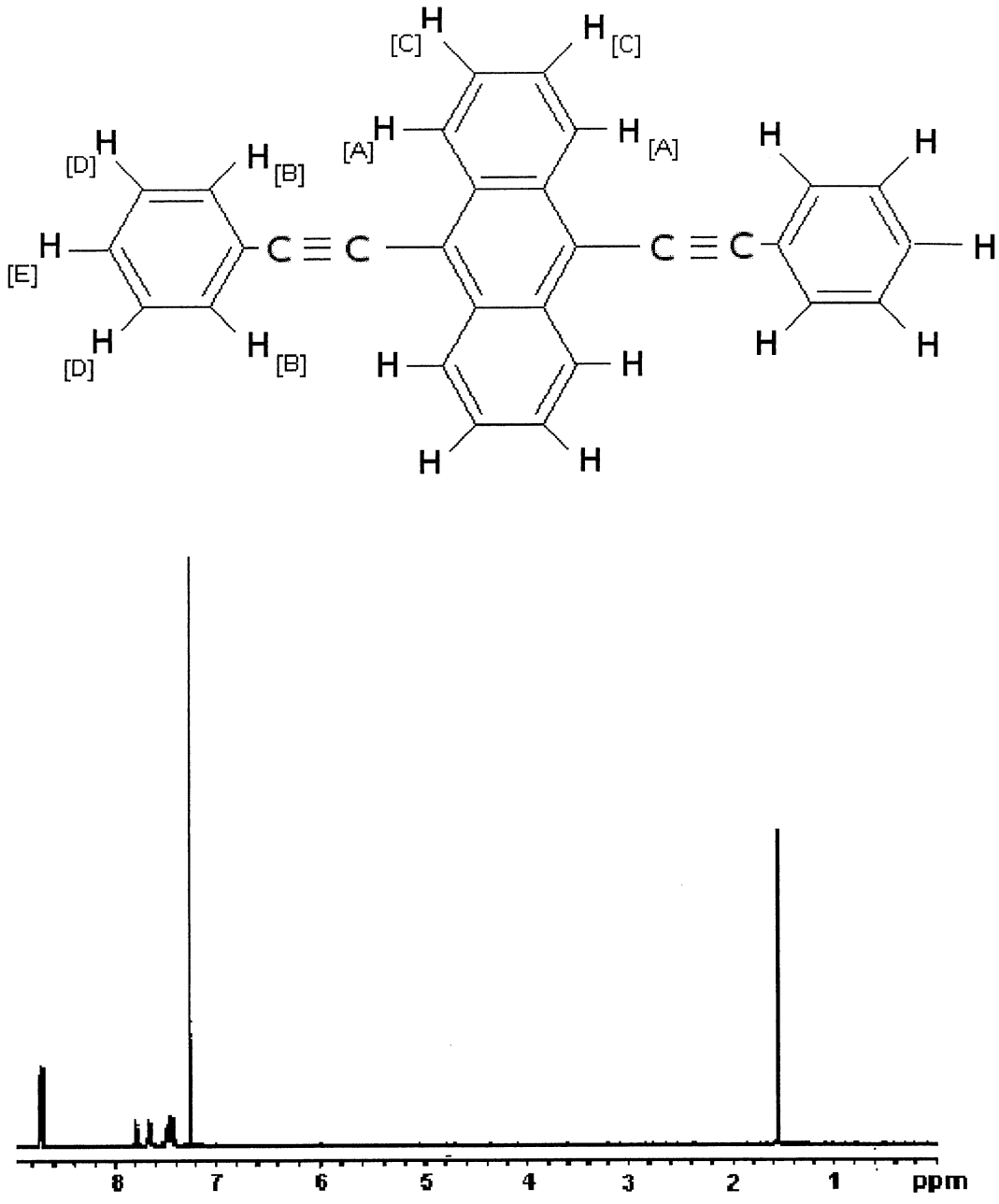


Figure 14. Proton NMR spectrum of 9,10-bis(phenylethynyl)anthracene.

3.2 9,10-bis(phenylethynyl)anthracene (BPEA)

Figure 14 shows the ^{1}H NMR spectrum obtained from 1mg of BPEA in 750 µl of deuterated chloroform ($CDCl_3$). Proton chemical shifts were referenced to the $CDCl_3$ peak at 7.2 ppm from TMS. Proton NMR spectrum revealed a total of 17 hydrogens , and these hydrogens are distributed in five different chemical environments thus giving five different chemical shifts. The downfield peak at 8.7 ppm represents four equivalent protons (H_A) attached to carbon atoms # 6. The peak at 7.9 ppm corresponds to four equivalent protons (H_B) attached to carbon atoms # 2. Similarly the peak at 7.64 ppm is attributed to four equivalent protons (H_C) on carbon atoms # 5. The peaks at 7.46 ppm and 7.43 ppm are due to two sets of four (H_D) and two protons (H_E) each attached to carbons 4 and 3 respectively (see Table 2). Thus it is evident that carbon atoms 1, 9 and 10 do not have any protons present and that the alkyne groups are in a non-terminal position.

The ^{13}C NMR spectrum of BPEA is shown in Figure 15. Carbon-13 shifts were referenced to the solvent $CDCl_3$ peaks at 76.91, 77.23 and 77.43 ppm from TMS. ^{13}C NMR spectrum analysis provides conclusive confirmation for the non-terminal C≡C structure. The two upfield ^{13}C peaks are clearly due to alkyne groups and the peak at 103 ppm can therefore be assigned to carbon # 9 and the peak at 87 ppm can be assigned to carbon # 12 [17]. The carbon atoms are numbered in the decreasing order of their carbon chemical shifts. The proton and carbon NMR spectrum chemical shifts for BPEA are summarized in Table 2.

Bonding of BPEA to silica hydride was successful only with Speier's catalyst. The FT-IR spectrum of this organic compound bonded to Kromasil hydride is shown in Figure 16. Because of the high surface area of the support material, the bands which resulted from the silanization and hydrosilation reactions are strong in intensity. However, the intensity of the Si-H stretching band at 2250cm^{-1} has decreased in comparison to the spectrum for Kromasil hydride. The appearance of new peak below 3000 cm^{-1} which represents aliphatic C-H stretching and the decrease in the Si-H band intensity all indicated the presence of BPEA on the hydride surface. The carbon-13 CP-MAS NMR spectrum of BPEA is shown in Figure 17. The peak at 19.5 ppm confirmed that the organic moiety is bonded to the surface. Possibly, one of the non-terminal alkyne groups of the BPEA molecule might be involved in the bonding but the exact structure of the bonded organic molecule cannot be elucidated as the amount of BPEA bonded to the hydride is very small as determined by elemental analysis (see Table 4).

3.3 Ethisterone

The proton NMR spectrum of ethisterone shown in Figure 18 is obtained from 1mg of sample in 750 µl deuterated chloroform, and all the chemical shifts were referenced to $CDCl_3$ peak at 7.2 ppm from TMS. The proton NMR spectrum revealed a total of 27 hydrogens. The peak at 2.58 ppm (H_B) corresponds to the hydrogen present on the terminal alkyne carbon # 6 (refer Figure 19). The chemical shifts of all the other protons have been summarized in Table 3.

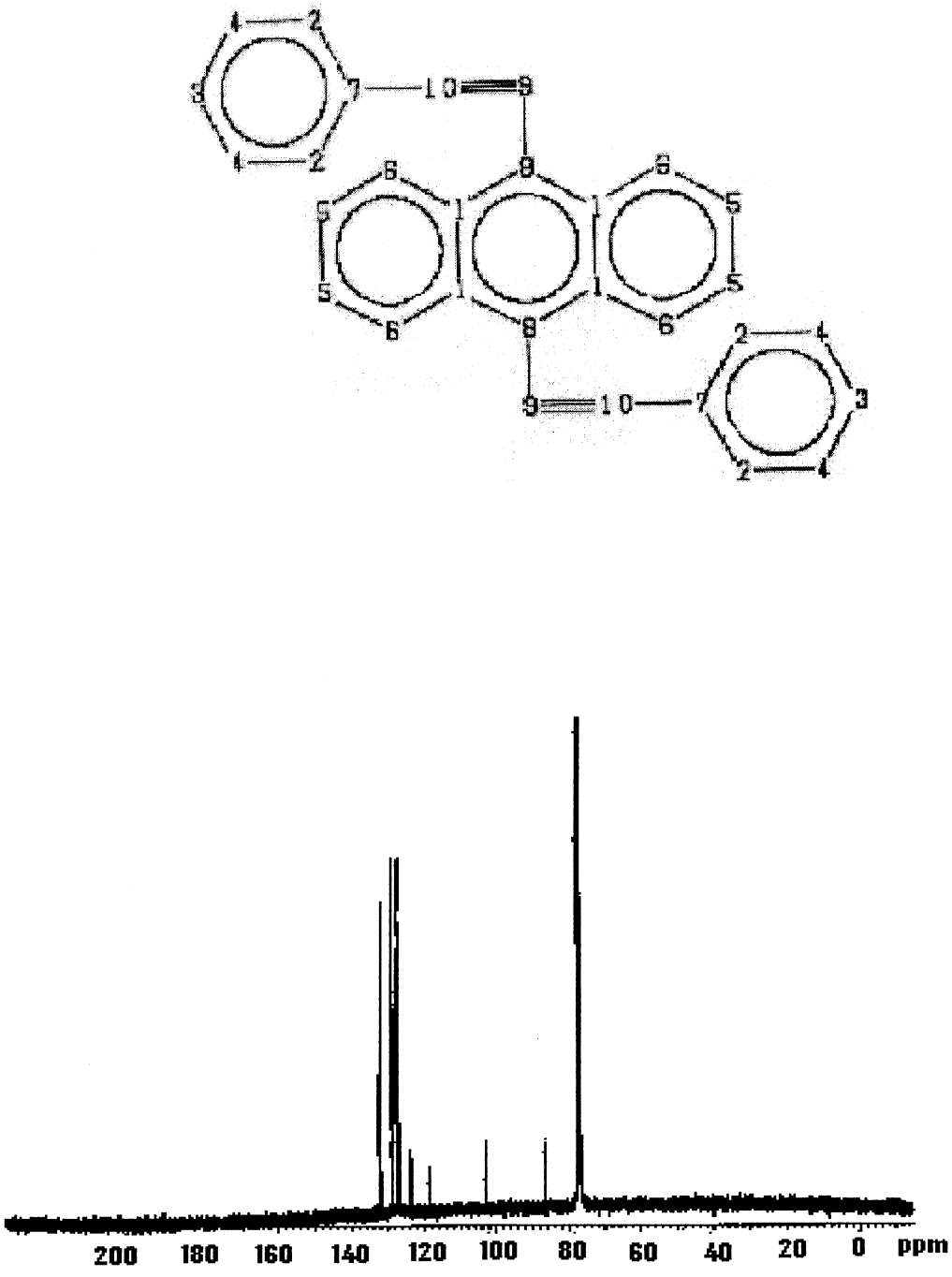


Figure 15. Carbon-13 NMR spectrum of 9,10-Bis(phenylethynyl)anthracene.

Table 2. Proton and Carbon-13 NMR chemical shifts for BPEA.

Proton NMR spectrum		***Carbon NMR spectrum***	
Hydrogen	ppm	Carbon	ppm
6($4H_A$)	8.7	1 (4C)	132.35
2($4H_B$)	7.79	2 (4C)	131.93
5($4H_C$)	7.64	3 (2C)	128.93
4($4H_D$)	7.46	4 (4C)	128.8
3($2H_E$)	7.43	5 (4C)	127.49
		6(4C)	127.05
		7 (2C)	124
		8 (2C)	119
		9 (2C)	102.9
		10(2C)	87

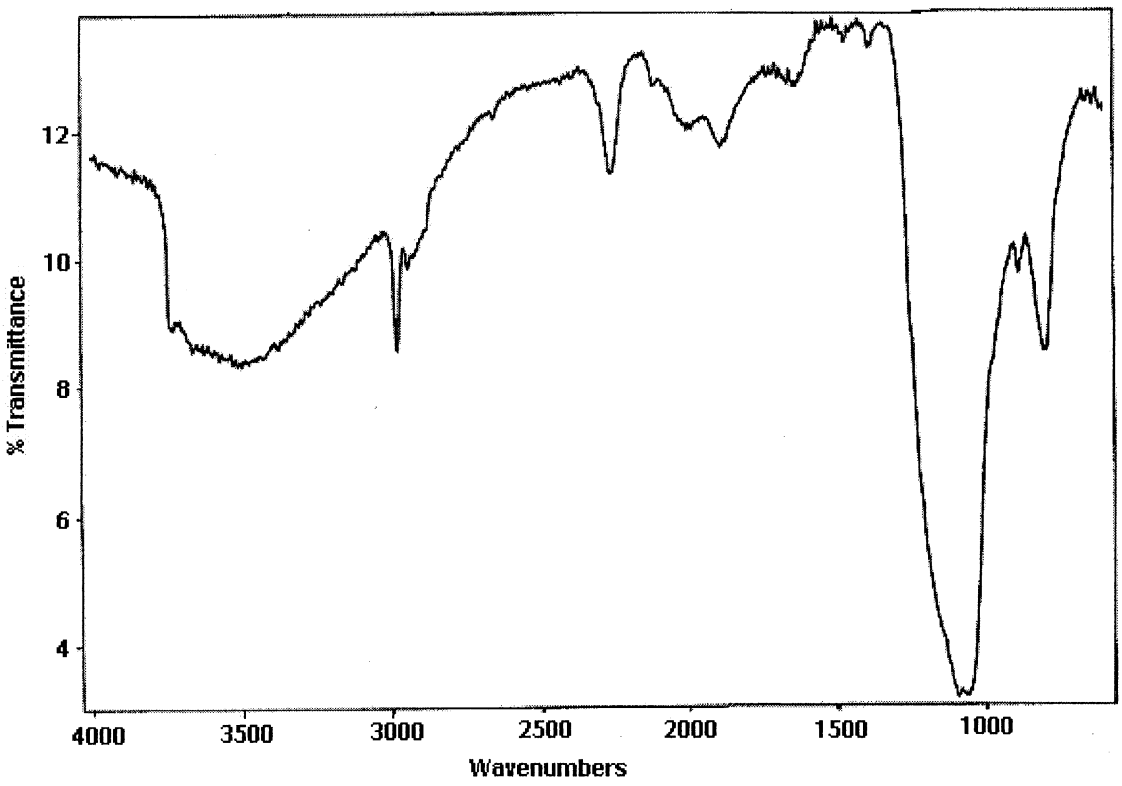


Figure 16. DRIFT spectrum of BPEA bonded to Kromasil hydride using Speier's catalyst.

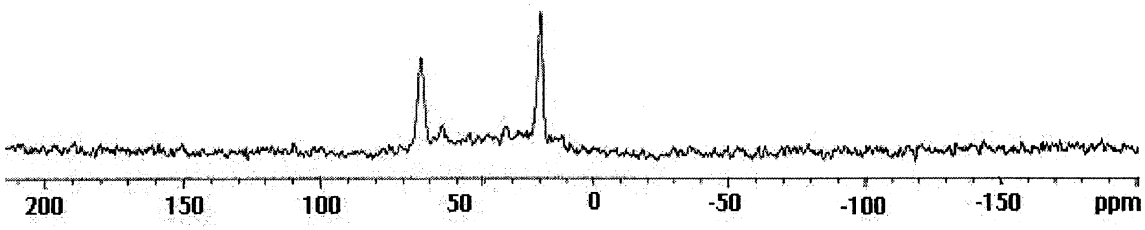


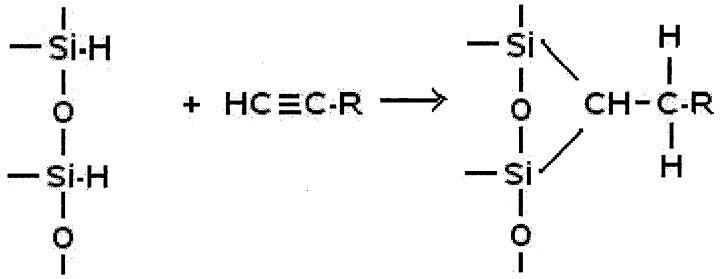
Figure 17. Carbon-13 CP-MAS spectrum of BPEA bonded phase.

The carbon-13 NMR spectrum of ethisterone is shown in Figure 19. The two slightly upfield chemical shifts at 87.86 and 74.17 ppm corresponds to the carbon atoms 4 and 6 respectively and this provides conclusive confirmation for the presence of terminal alkyne carbons. All the other carbons have been assigned chemical shifts which are summarized in Table 3.

Bonding of ethisterone to Kromasil hydride was successful using both Speier's catalyst and t-butyl peroxide. Figure 20 shows the DRIFT spectrum of ethisterone bonded phase using Speier's catalyst. In this spectrum, the aliphatic C-H stretching vibration at 3000 cm^{-1} and the Si-H peak at 2250 cm^{-1} with reduced intensity confirms the bonding. The hydrosilation product of ethisterone using t-butyl peroxide also gave a similar DRIFT spectrum which is shown in Figure 21. DRIFT provided an indirect method for confirming the actual bonding of an organic moiety as opposed to adsorption on the surface.

The carbon-13 CP-MAS NMR spectrum confirms the bonding and also provides structural information about the molecule bonded to the surface. Figure 22 shows the ^{13}C solid state NMR spectrum for the ethisterone bonded phase using Speier's catalyst. The peak at 11.4 ppm represents a carbon atom bonded to a silicon (Si-C) and the only point of attachment of the organic molecule to the hydride surface is through the terminal alkyne carbon. The peak at 28.7 ppm corresponds to the methylene carbon that is present adjacent to the bonded carbon atom (Si-C-C*). The presence of the methylene group indicates the possibility of a double Si-C linkage between the surface and the bonded

organic moiety. One such bonded material which might result in a high degree of stability is shown below [11].



where 'R' represents the rest of the ethisterone steroid molecule.

The peaks at 44 ppm and 55 ppm corresponds to the carbon atoms 10 and 7 respectively (carbon atoms numbered in the decreasing order of their ^{13}C chemical shifts). A very weak peak at 164 ppm is due to the olefin group present in the steroid skeleton and a ketone functional group in the organic moiety gave a weak signal at 209 ppm. The ^{13}C CP-MAS NMR spectrum of the ethisterone bonded phase using t-butyl peroxide also gave the similar results which are shown in Figure 23. The peak at 12 ppm confirmed the actual bonding of the organic molecule to the surface. The methylene group gave the peak at 35 ppm and the peak at 54 ppm corresponds to carbon atom #7.

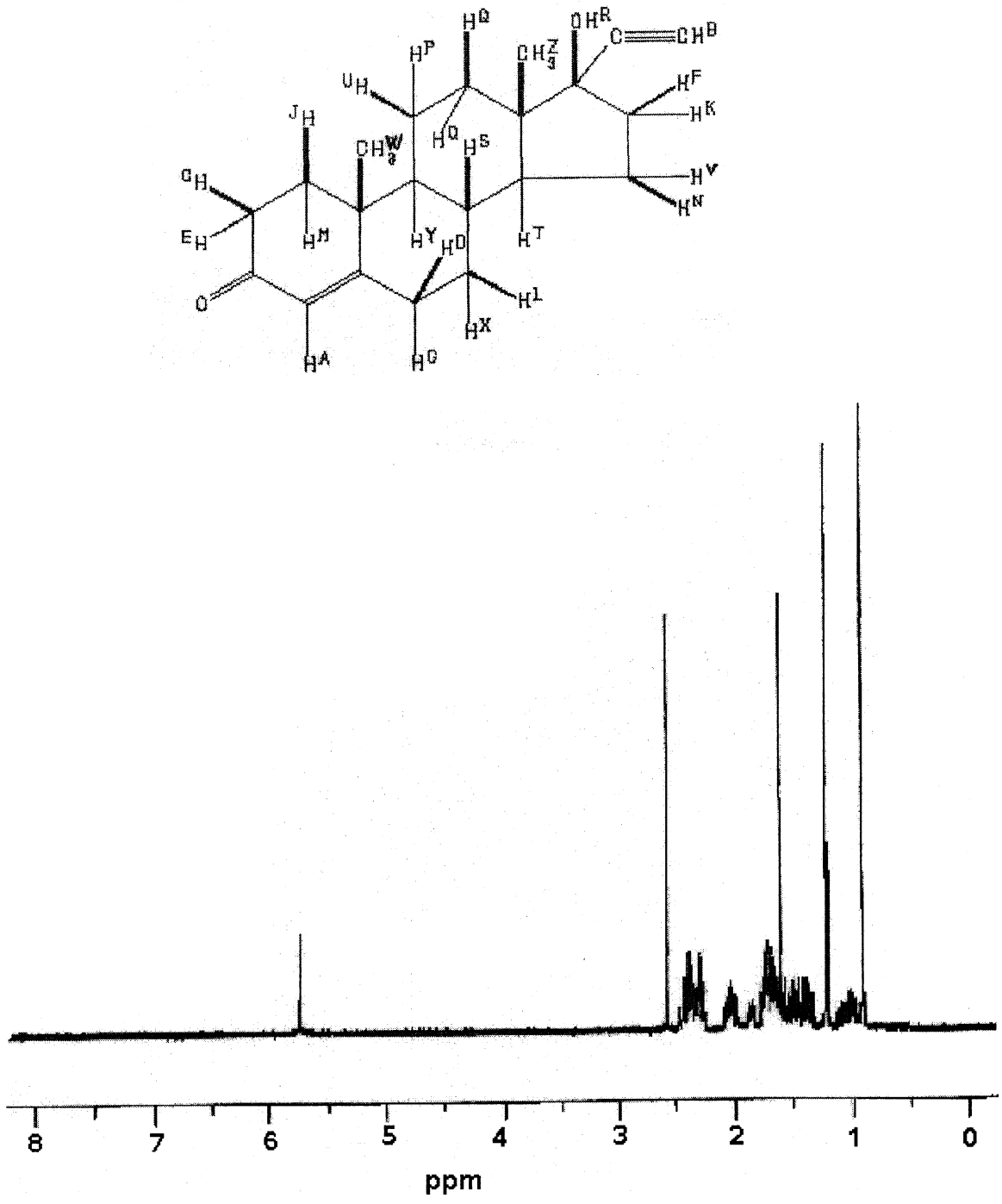


Figure 18. Proton NMR spectrum of ethisterone.

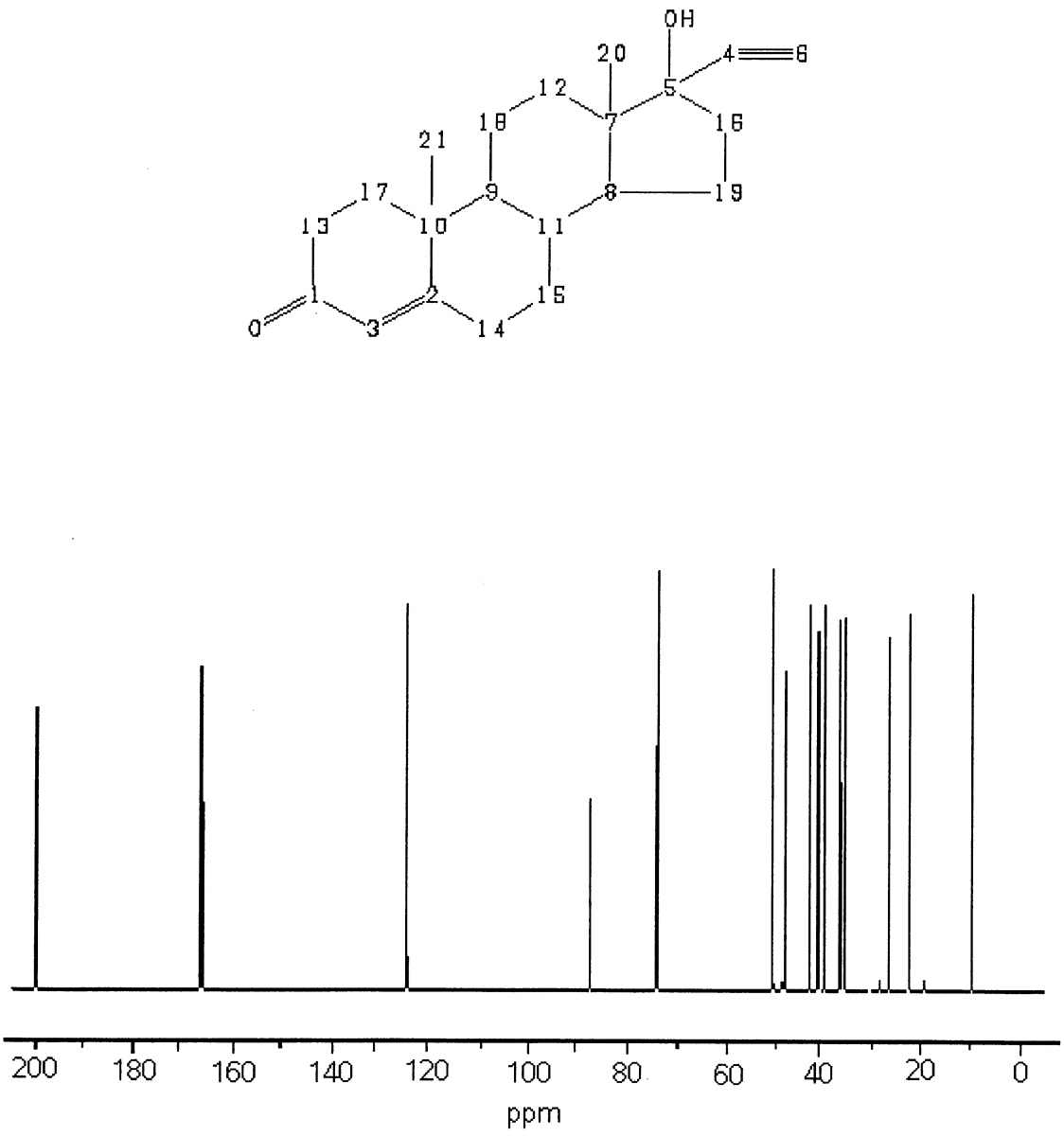


Figure 19. Carbon-13 NMR spectrum of ethisterone.

Table 3. Proton and carbon-13 NMR chemical shifts for ethisterone.

Proton NMR spectrum		***Carbon NMR spectrum***	
Hydrogen	ppm	Carbon	ppm
3 ($1H_A$)	5.73	1 (1C)	199.72
6 ($1H_B$)	2.58	2 (1C)	166.38
13 ($1H_C$)	2.43	3 (1C)	124.68
14 ($1H_D$)	2.39	4 (1C)	87.86
13 ($1H_E$)	2.36	5 (1C)	81.47
16 ($1H_F$)	2.31	6 (1C)	74.17
14 ($1H_G$)	2.28	7 (1C)	50.84
17 ($1H_J$)	2.05	8 (1C)	49
16 ($1H_K$)	2.01	9 (1C)	48.03
15 ($1H_L$)	1.85	10 (1C)	42.57
17 ($1H_M$)	1.73	11 (1C)	41.02
19 ($1H_N$)	1.7	12 (1C)	39.65
18 ($1H_P$)	1.66	13 (1C)	36.53
12 ($1H_Q$)	1.75 – 1.63	14 (1C)	35.56
OH ($1H_R$)	1.61	15 (1C)	30.78
11 ($1H_S$)	1.59	16 (1C)	28.53
8 ($1H_T$)	1.49	17 (1C)	26.63
18 ($1H_U$)	1.43	18 (1C)	26.31
19 ($1H_V$)	1.36	19 (1C)	22.48
21 ($3H_W$)	1.2	20 (1C)	10.12
15 ($1H_X$)	1.06	21 (1C)	9.58
9 ($1H_Y$)	0.98		
20 ($3H_Z$)	0.9		

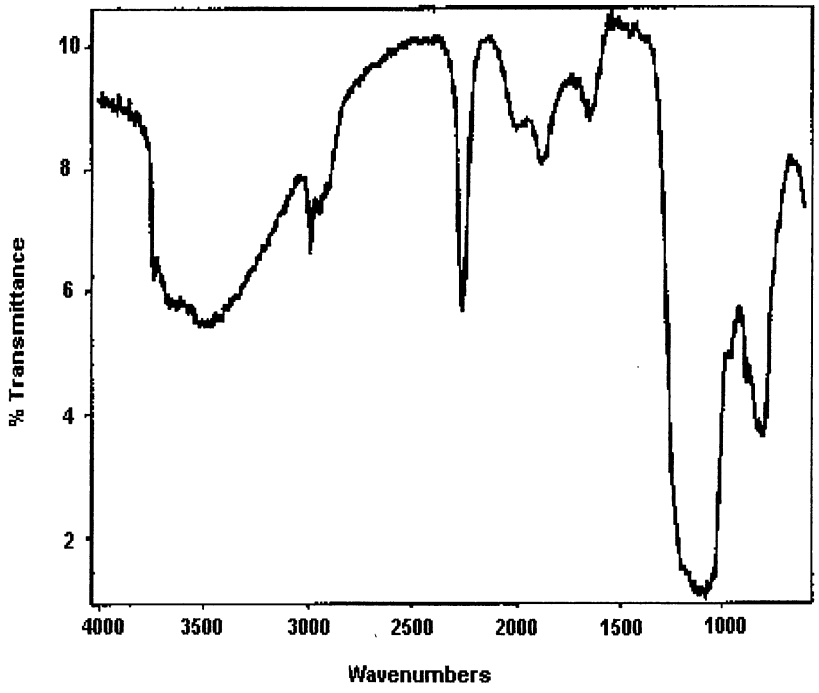


Figure 20. DRIFT spectrum of ethisterone bonded to Kromasil hydride using Speier's catalyst.

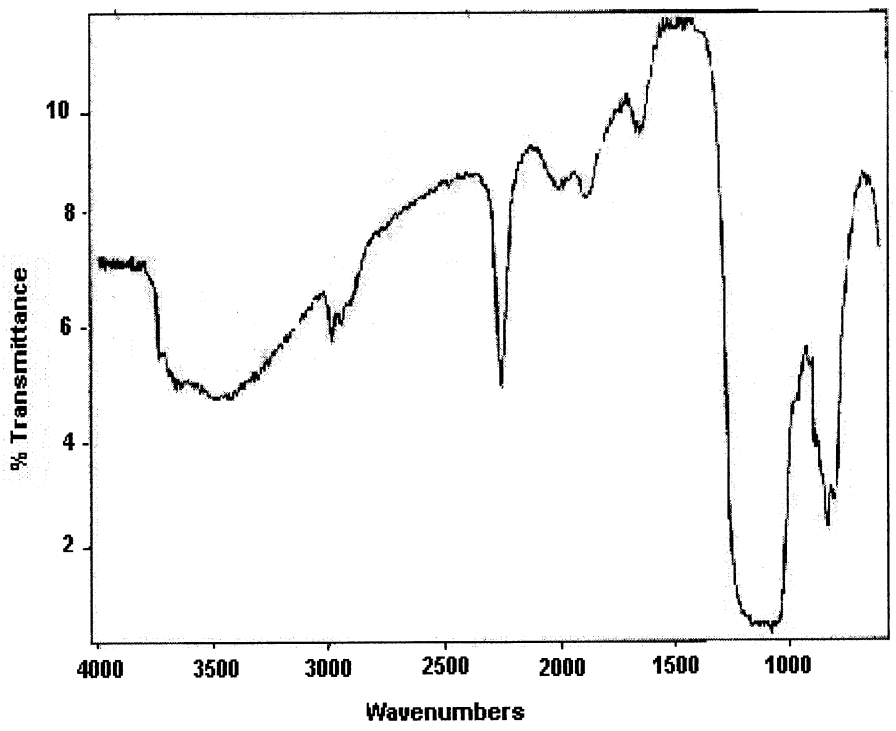


Figure 21. DRIFT spectrum of ethisterone bonded to Kromasil hydride using t-butyl peroxide.

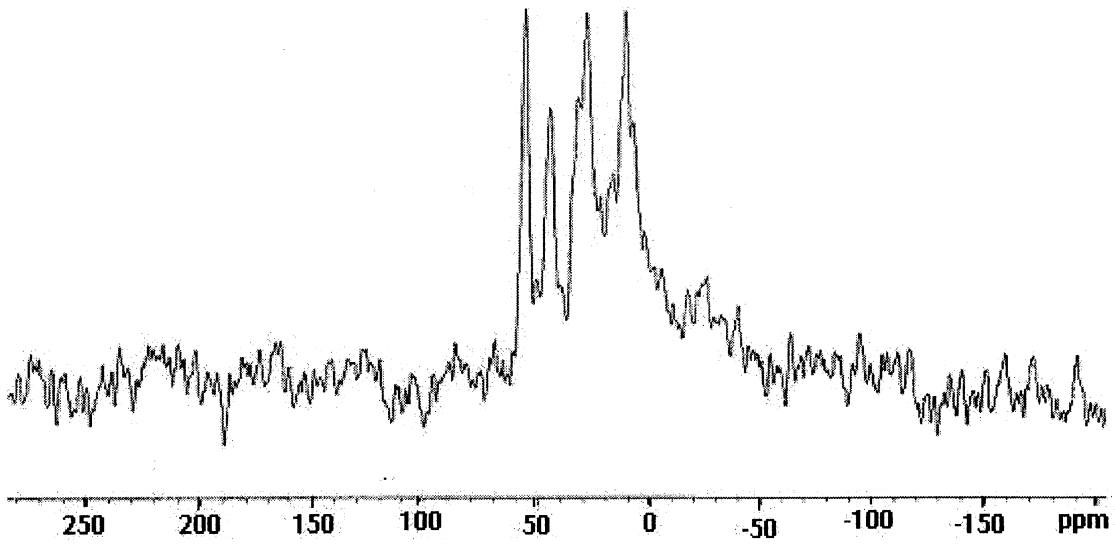


Figure 22. Carbon-13 CP-MAS spectrum of ethisterone bonded phase using Speier's catalyst.

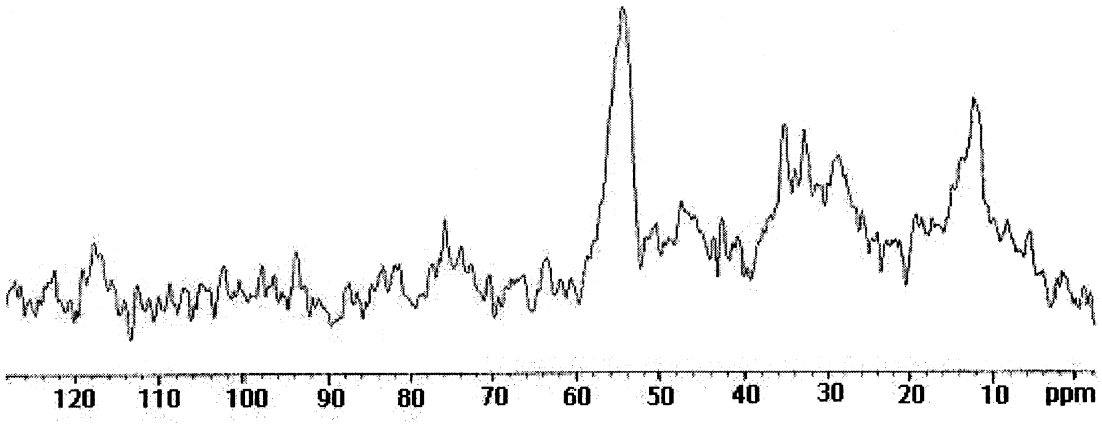


Figure 23. Carbon-13 CP-MAS NMR spectrum of ethisterone bonded phase using t-butyl peroxide.

3.4 Bonded Phases

The concentration of surface-bonded groups, α_R , was calculated from the carbon content of the bonded material determined by elemental analysis and the BET specific surface area of the native silica before bonding. The following equation proposed by Berendsen and de Galan was used to determine the amount of bonded material on the surface on the basis of $\mu mol/m^2$:

$$\alpha_R\,(\,\mu mol/m^2\,) = 10^6\,\rho_c / (10^2 M_c n_c - \rho_c M_R)\, S_{BET} \qquad (5)$$

where ρ_c is the carbon percentage of the bonded material (by weight after correction for any carbon present before bonding), M_c is the atomic weight of the carbon, n_c is the number of carbon atoms in the bonded organic group, M_R is the molecular weight of the attached group, and S_{BET} is the specific surface area of the native substrate [4]. The elemental analysis and the surface coverage for the various bonded silica-based materials in this study are shown in Table 4.

The hydrosilation of ethisterone using a Pt catalyst gave a lower yield on the hydride surface when compared to the bonded phase synthesis using the free radical catalyst, t-butyl peroxide as shown by elemental analysis. This is because the transition step in the attachment of the organic moiety to the hydride surface involves forming a complex with the catalyst. In the case of hexachloroplatinic acid, the intermediate species can be of considerable size and this might have resulted in a lower yield of organic

species on the surface [14].

Table 4. Elemental analysis and the surface coverage of the bonded phases in this study.

COLUMN	*%CARBON*	*SURFACE COVERAGE*
9,10-BPEA	2.44%	0.162 μMol/m^2
Ethisterone (Pt)	1.28%	0.09 μMol/m^2
Ethisterone (Fr.rad)	2.05%	0.18 μMol/m^2

3.5 HPLC Performance

3.5.1 Tocopherols

3.5.1.1 Separations in the Reversed-phase Mode

In order to characterize and compare the newly synthesized bonded phases, some preliminary chromatographic tests using d-α , dl-α and d-δ tocopherols as the test solutes were performed on all four columns (9-vinyl anthracene (fr.rad), 9,10BPEA (Pt), ethisterone (Pt) and ethisterone (fr.rad)). Elution of the individual solutes was done first using four different mobile phase compositions of methanol and water under isocratic conditions and finally separations of the mixture of the three tocopherols was performed.

Generally, in the reverse phase conditions, tocopherol follows the elution order given below with the most polar solute eluting first : d- δ < dl- α ~ d- α tocopherols and increasing the polarity of the mobile phase has the effect of increasing the elution time. The d- δ tocopherol with only one methyl substituent on the tocol ring is the most polar of

the 3 tocopherols and is expected to elute first. The next in the elution order has to be the synthetic dl-α-tocopherol which is a racemic mixture of eight stereoisomers of α-tocopherol and, d-α- tocopherol having three methyl substituents on the tocol ring.

9-vinylanthracene provided no significant separation of the three tocopherols and increasing the polarity of the mobile phase from methanol (100%) to methanol :water (85:15) resulted in little effect on the mixture separation. The retention of the tocopherols on all the new stationary phases was also very small as the solutes in the mixture were eluted in less than 3 minutes. If the polarities of the mobile phase and solutes are matched but different from that of the stationary phase , then the stationary phase cannot compete successfully for the sample components [12]. This is likely the reason for the short retention times of the tocopherols on the new phases.

The 9,10-bis(phenylethynyl)anthracene (BPEA) bonded phase column gave very high back pressure while operating under reversed-phase chromatographic conditions. By slightly increasing the polarity of the methanol/water mobile phase, the stationary phase, turned dark and any further HPLC separations became unsuccessful with this column. The Pt catalyst which was used in the bonded phase synthesis might have complexed with the organic material and itself might have been reduced during HPLC operation leading to the dark color of the bonded phase [15]. The DRIFT of 9,10 BPEA bonded phase after use shown in Figure 24 supports the above reason. The Si-H stretching band at 2250 cm^{-1} is much less intense and the strong C-H stretching vibration between 3000 and 2800 cm^{-1} is diminished in intensity but still indicates the presence of bonding.

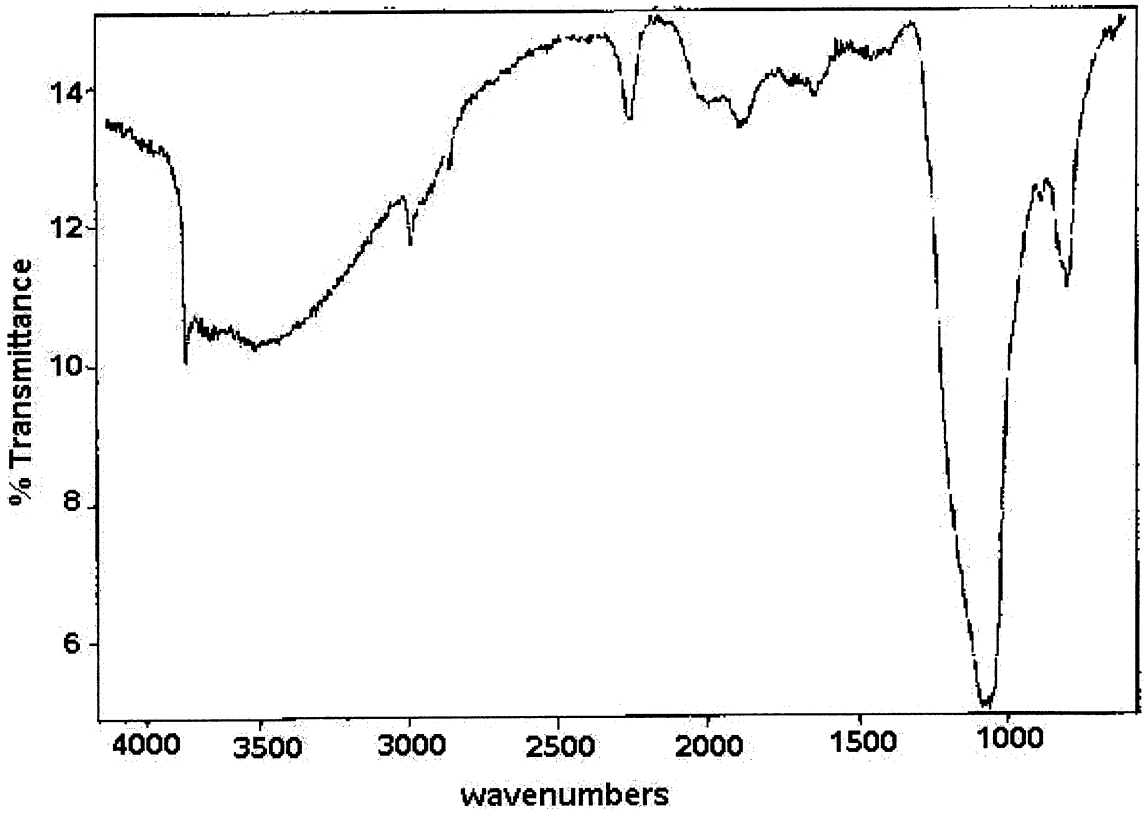


Figure 24. DRIFT spectrum of 9,10 BPEA bonded phase after testing.

Another interesting feature that was observed was the appearance of the OH stretching band at 3750 cm^{-1} that corresponds to the exposed unreacted silanol groups to the surface.

The ethisterone (Pt) and ethisterone (fr.rad) columns also proved unsuccessful in the separation of the tocopherols and increasing the mobile phase polarity had no effect on the separation. Figure 25 shows the comparison of the average retention time as a function of methanol % in the 3 columns, 9-vinylanthracene (fr.rad), ethisterone (Pt) and ethisterone (fr.rad) respectively using d-α-tocopherol as the reference. The 3 columns exhibited different average retention times under four different mobile phase compositions. 9-vinylanthracene (fr.rad) showed an initial increase (using methanol:water(95:5)) but final decrease in the average retention times with increased mobile phase polarity. Ethisterone (Pt) displayed an initial increase in the average retention time using methanol/water in the ratios 95:5 and 90:10, but later (using methanol/water in the ratio 85:15), the average retention time was stable. Whereas ethisterone (fr.rad) displayed an initial increase in the average retention time (using methanol:water(95:5)). Later, the average retention time was slightly decreased(using methanol:water(90:10)) but finally was increased with increased mobile phase polarity (using methanol:water (85:15)). These observations shows that all the 3 columns are inconsistent in the reversed-phase mode.

3.5.1.2 Separations in the Normal Phase

The separation of tocopherols was successful using 100% hexane as the mobile

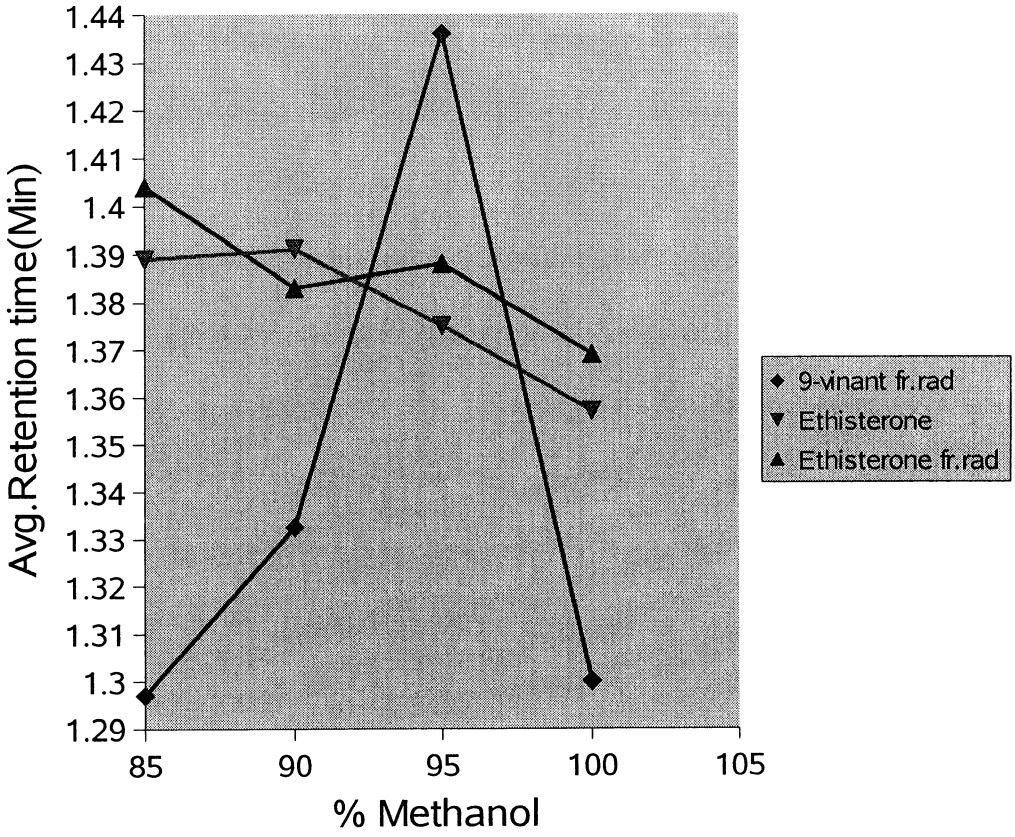


Figure 25. Comparison of retention behavior of the 3 columns using d-α tocopherol as the reference in the reversed-phase mode.

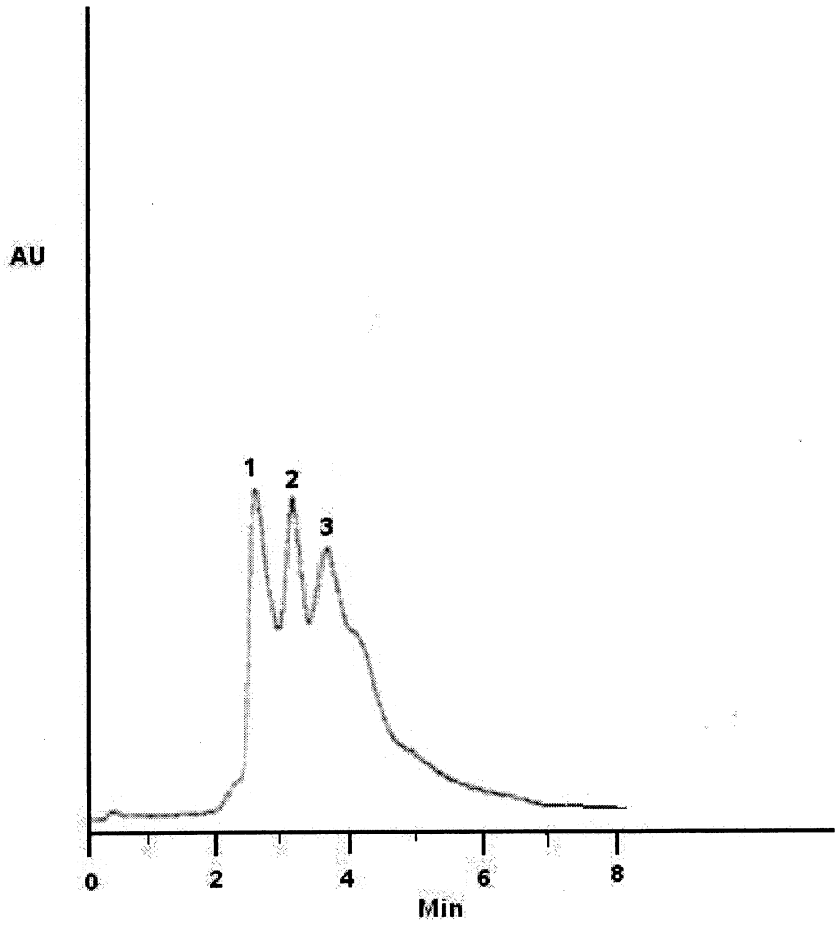


Figure 26. Typical HPLC separation profile for the tocopherol mixture on the 9-vinyl anthracene column. The mixture was separated isocratically using 100% hexane at a flow rate of 2ml/min and detected at 290 nm. Component identification: 1, d-α tocopherol (2.76min); 2, dl-α tocopherol (3.28min); 3, d-δ tocopherol (3.73min).

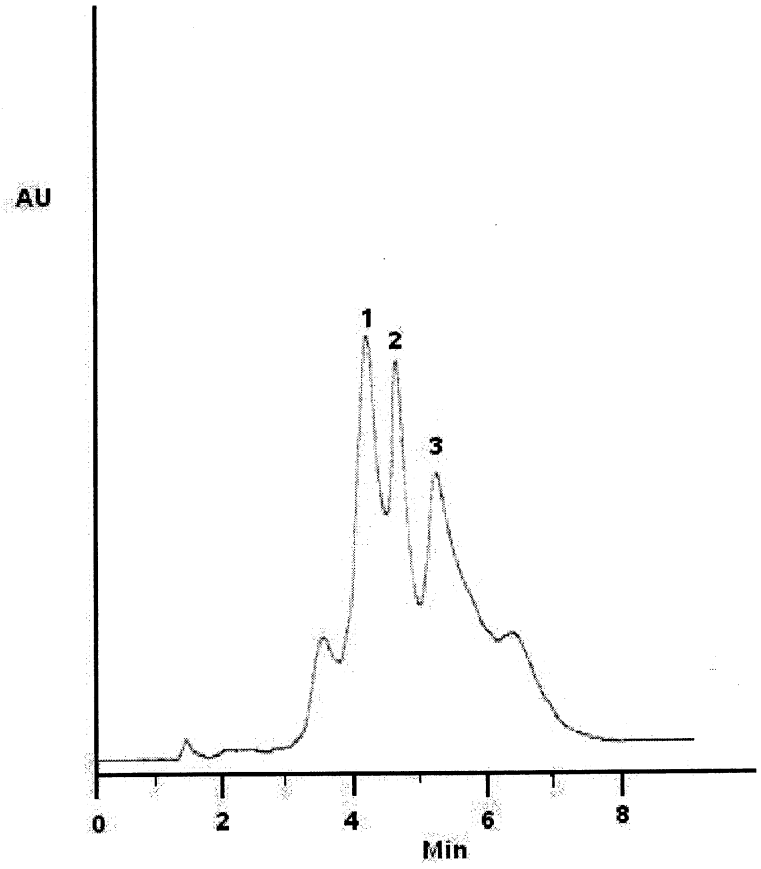


Figure 27. Typical HPLC separation profile for the tocopherol mixture on the ethisterone (Pt) column. Component identification and separation conditions are the same as in Figure 26.

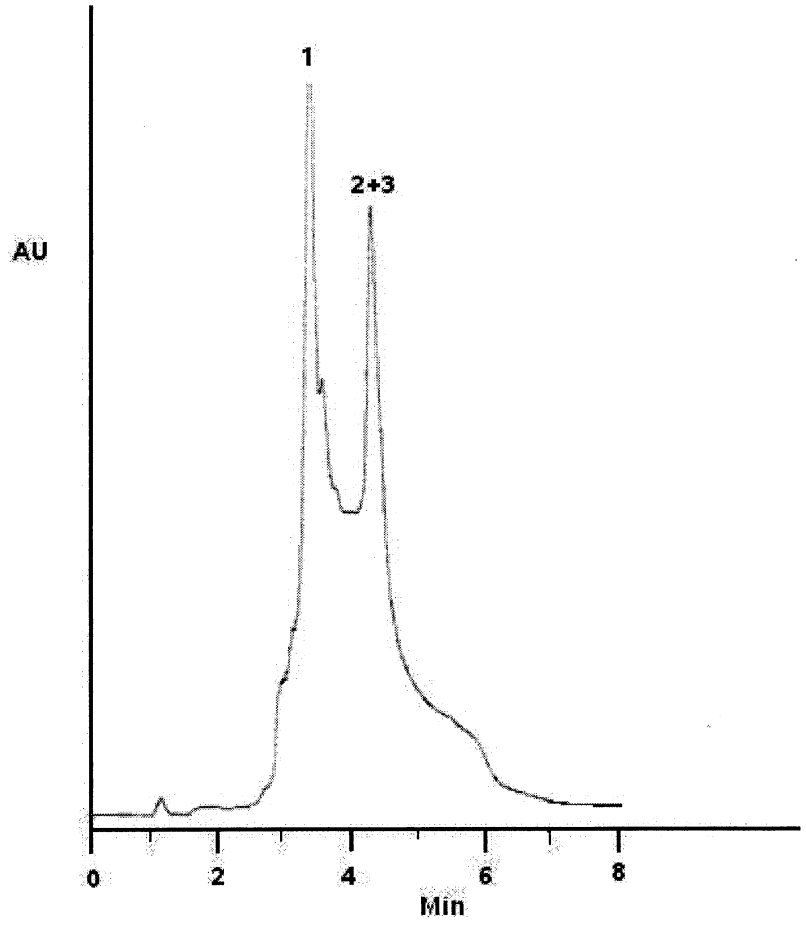


Figure 28. Typical HPLC separation profile for the tocopherol mixture on the ethisterone (fr.rad) column. Component identification and separation conditions are the same as in Figure 26.

phase although there was no significant baseline separation. An elution order of d-α < dl-α < d-δ tocopherol was observed in two of the three columns. Figures 26, 27, 28 show typical chromatograms for the mixture of tocopherols on different bonded phases. Three chromatographic peaks showing the separation of the three tocopherols were observed on 9-vinylanthracene (fr.rad) and ethisterone (Pt) columns. Using ethisterone (fr.rad) column, only 2 peaks were observed and dl-α and d-δ tocopherols were co-eluted.

Comparison of the 3 columns using the average retention time of d-α tocopherol as the reference plotted against four different mobile phase compositions of hexane/amyl alcohol is shown in Figure 29. The three columns displayed a similar trend of a decrease in the retention times with decreased % hexane (increased polarity) in the hexane/amyl alcohol mobile phase and were consistent with the normal phase behavior.

3.5.2 Polycyclic Aromatic Hydrocarbons

3.5.2.1 Separations in the Reversed-phase

To evaluate the retention characteristics of the novel stationary phases, the following low molecular weight PAHs were used as the test solutes: naphthalene, anthracene, acenaphthene and fluorene at 1mg/ml in acetonitrile. All of the above solutes were run separately so that their individual retention times could be determined and later separations of the mixture were performed. Generally, in the reverse phase an elution order of naphthalene < acenaphthene < fluorene < anthracene is observed. However, the three columns showed no separation of PAHs and increasing the polarity of the mobile

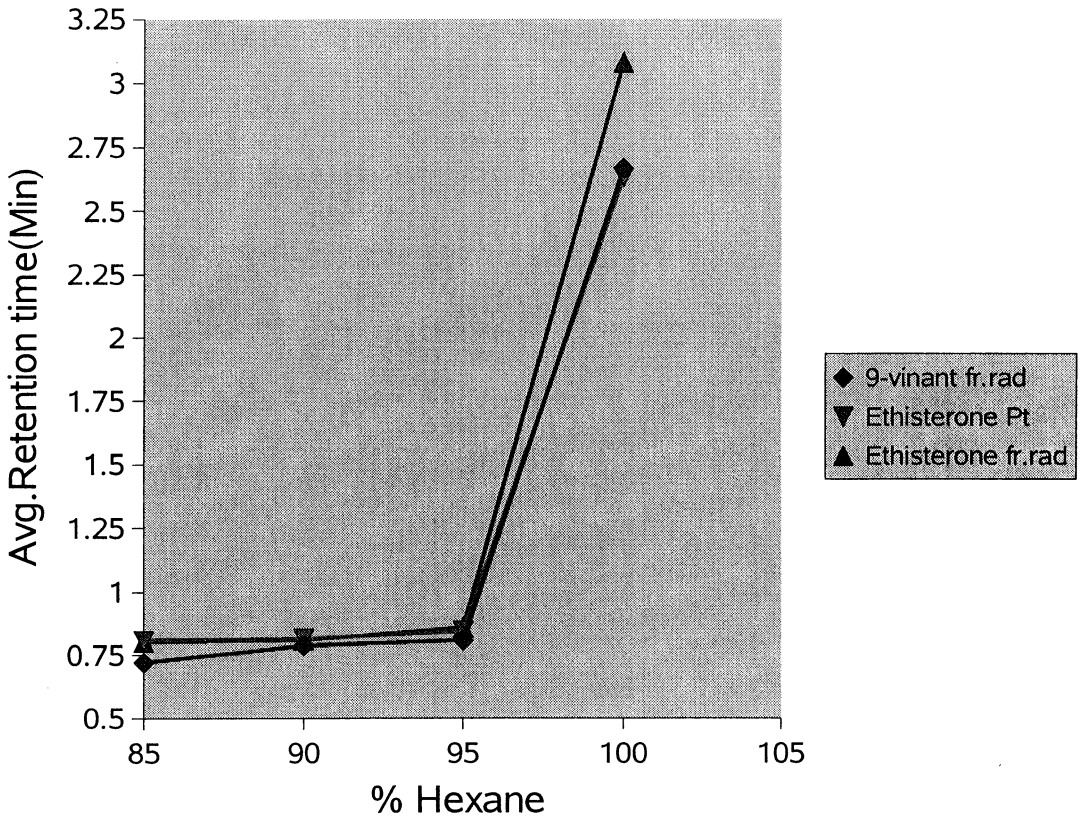


Figure 29. Comparison of retention behavior of the 3 columns using d-α tocopherol as the reference in the normal phase.

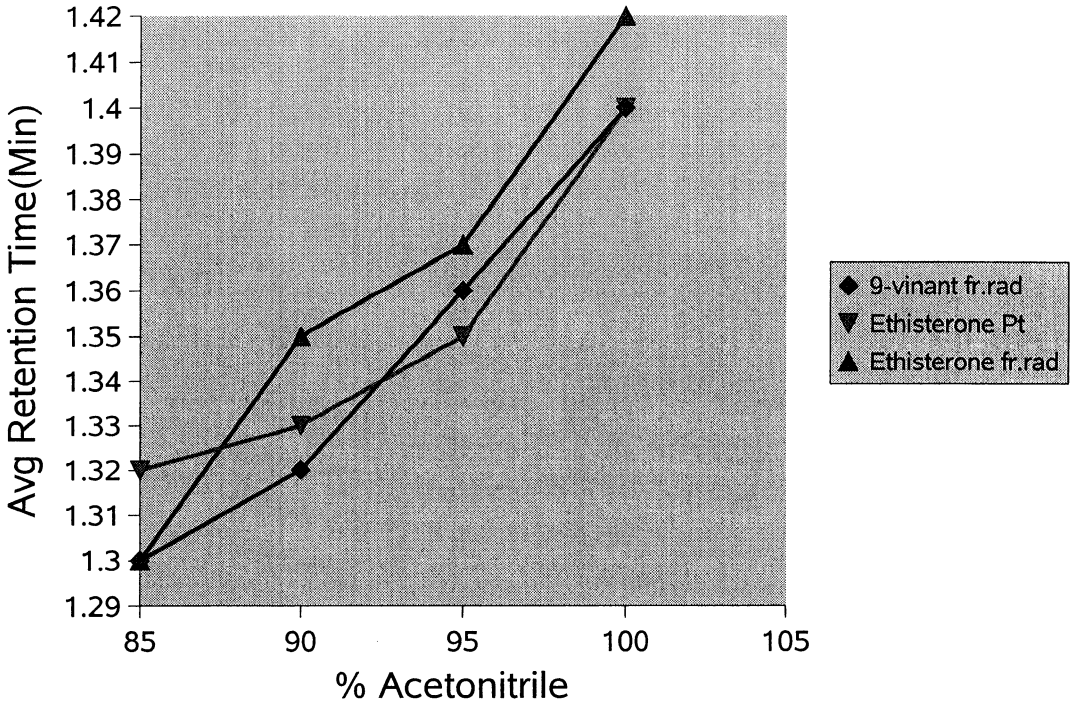


Figure 30. Comparison of retention behavior of the 3 columns using naphthalene as the reference in the reversed-phase.

phase to acetonitrile:water (85:15) had no effect on the separation. Figure 30 shows a comparison of the retention behavior of the 3 columns using naphthalene as the reference at four different mobile phase compositions. The 3 columns displayed almost similar trend of decrease in the average retention time with increasing mobile phase polarity which is not consistent with the reversed-phase behavior.

3.5.2.2 Separations in the Normal Phase

The newly synthesized stationary phases exhibited some selectivity towards the PAHs in the normal phase when compared to that in the reversed-phase. At ambient column temperatures and using 100% hexane as the mobile phase, a partial separation of the mixture of PAHs was observed.

The 3 columns eluted naphthalene and acenaphthene as one chromatographic peak and anthracene and fluorene were eluted as another peak but with different retention times. Figures 31, 32, 33 show typical chromatograms for the mixture of PAHs mentioned above. The mechanism of retention of these small PAHs seems to be dependent on the shape recognition and the extent of π- π interactions with the stationary phases [18]. Naphthalene and acenaphthene with almost similar molecular shapes interacted less with the stationary phase and were eluted earlier at the same retention time. Anthracene and fluorene appear to have exhibited extended π- π interactions with the stationary phase and so were retained longer. The same order of elution was followed in all 3 columns thus supporting the above hypothesis.

Figure 34 shows a comparison of the retention behavior of the 3 columns using naphthalene as the reference. Here all the 3 columns displayed a pattern of decrease in the average retention times as the percentage of hexane was decreased (increased polarity)in the hexane /chloroform mobile phase which is consistent with the normal phase behavior.

3.5.3 Steroids

3.5.3.1 Separations in the Reversed-phase

All the test solutes that were selected consisted of a common steroid skeleton and with varying number of polar groups (ketone, hydroxyl groups etc.). First, the individual retention times of all the selected steroids were determined and finally the mixture of steroids at 1mg/ml in acetonitrile was run isocratically. Generally, an elution order of corticosterone < estradiol < prednisone < Estrone < adrenosterone < 11- α acetoxy progesterone is expected in the reverse phase columns with the highly polar solutes eluting first.

Using 100% acetonitrile as the mobile phase, 9-vinylanthracene (fr.rad) column gave a partial separation of 6 components as three chromatographic peaks. The solvent acetonitrile is also called aqueous normal phase solvent as it is not completely organic. It contains 1% water and it does have effect on the chromatographic separations. On this column, estradiol eluted first at 1.36 minutes. The next in the elution order were 11-α acetoxy progesterone, adrenosterone and estrone at 1.62 minutes.

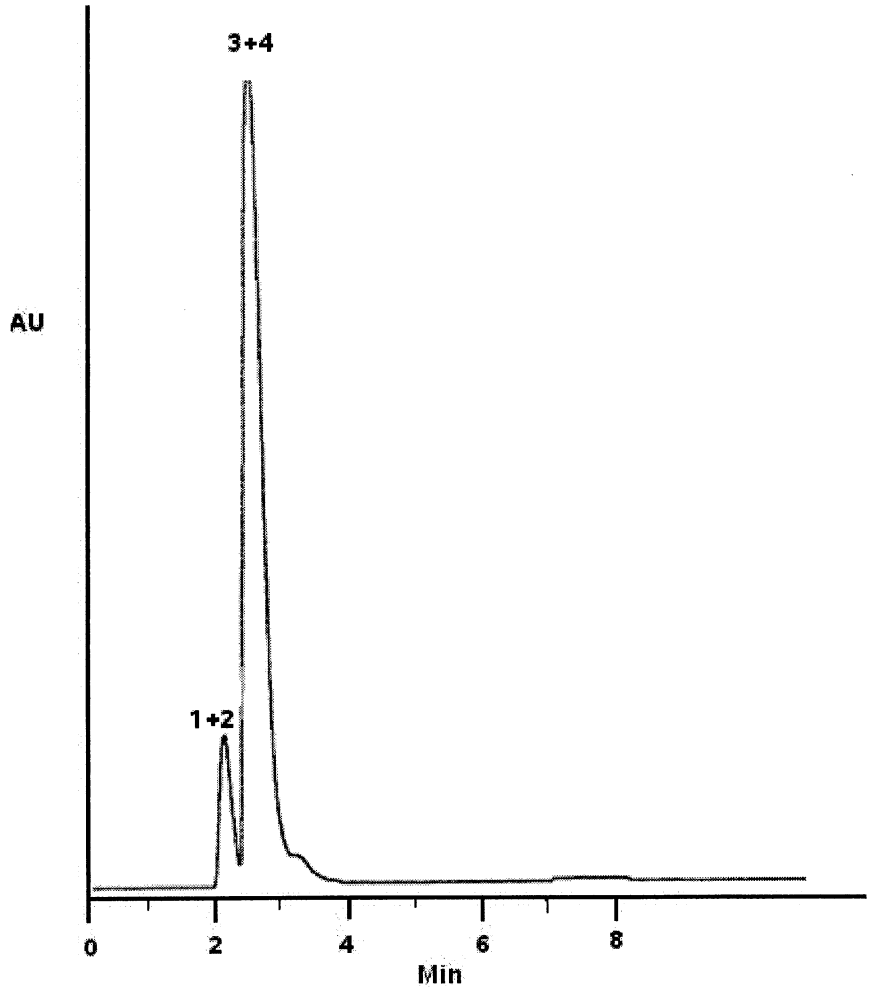


Figure 31. Typical HPLC separation profile for the PAH mixture on the 9-vinylanthracene (fr.rad) column. The mixture was separated isocratically using 100% hexane at a flow rate of 1ml/min and detected at 254 nm.Component identification: 1, naphthalene; 2, acenaphthene; 3, anthracene; 4, fluorene.

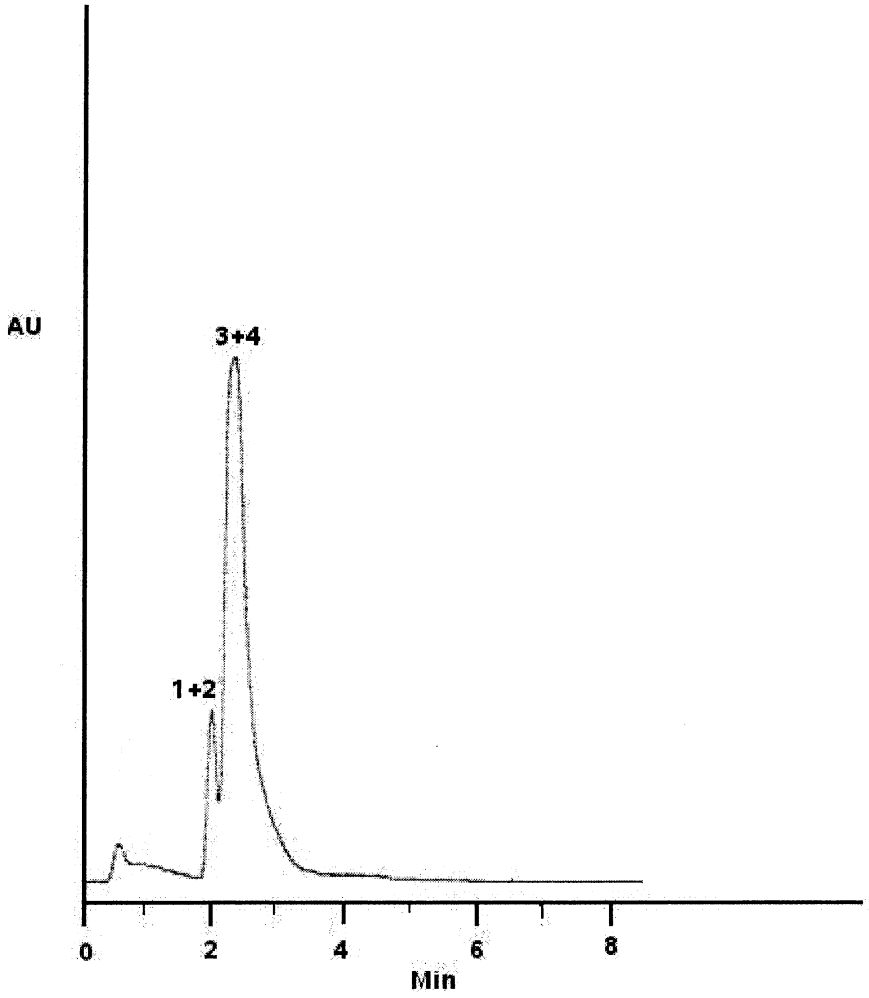


Figure 32. Typical HPLC separation profile for the PAH mixture on the ethisterone (Pt) column. The component identification and separation conditions are the same as in Figure 31.

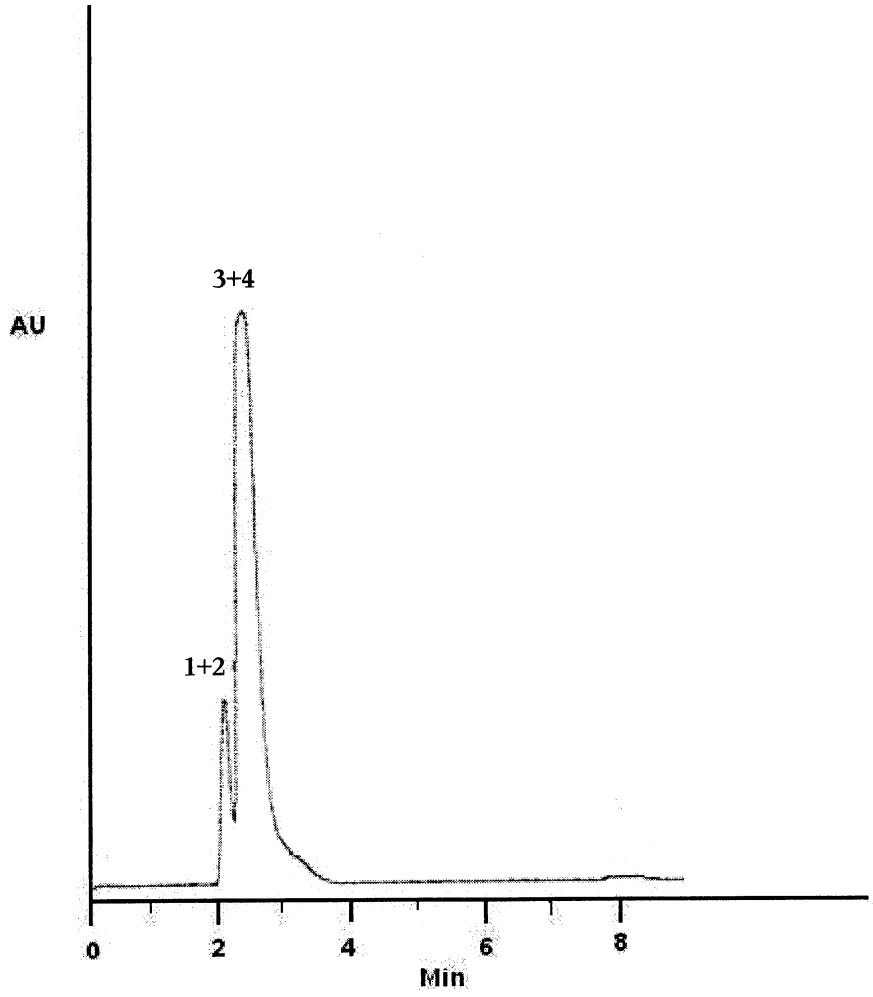


Figure 33. Typical HPLC separation profile for the PAH mixture on the ethisterone (fr.rad) column. The component identification and separation conditions are the same as in Figure 31.

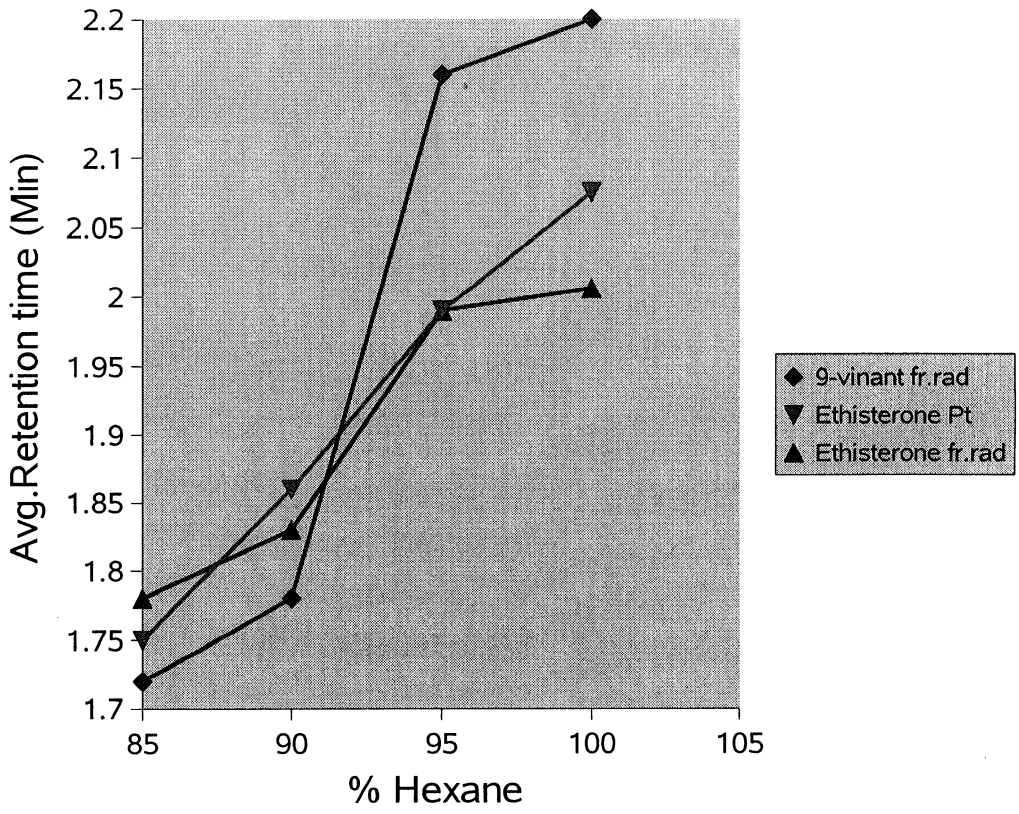


Figure 34. Comparison of the retention behavior of the 3 columns using naphthalene as the reference in the normal phase.

Finally, corticosterone and prednisone were eluted as one peak at 2.51 minutes. The ethisterone (Pt) and ethisterone (fr.rad) columns gave a different mixture separation. Corticosterone, estradiol and estrone all eluted as one chromatographic peak. 11-α acetoxy progesterone, and adrenosterone had the same retention times and therefore eluted as one peak. The final test solute that was eluted was prednisone. Figures 35,36,37 shows the typical chromatograms for mixture separation of steroids. The mixture separation was repeated three times to ensure reproducibility of results. The newly synthesized columns displayed no definite pattern in the order of elution when using different mobile phase compositions of acetonitrile/water. Figure 38 shows a comparison of the 3 columns using corticosterone as the reference. All the columns displayed a decrease in the average retention time with increasing mobile phase polarity (i.e., decreasing % acetonitrile in the the mobile phase). This proves that the 3 columns do not possess the desired reversed-phase characteristics.

3.5.3.2 Separations in the Normal Phase

None of the 3 columns appeared to furnish effective separation of the mixture of steroids using four different mobile phase compositions of hexane/methylene chloride in the normal phase mode. Figure 39 which shows the retention behavior of the 3 columns with corticosterone as the reference indicated a decrease in the average retention time with increasing mobile phase polarity. This shows that all the columns under study are exhibiting normal phase characteristics.

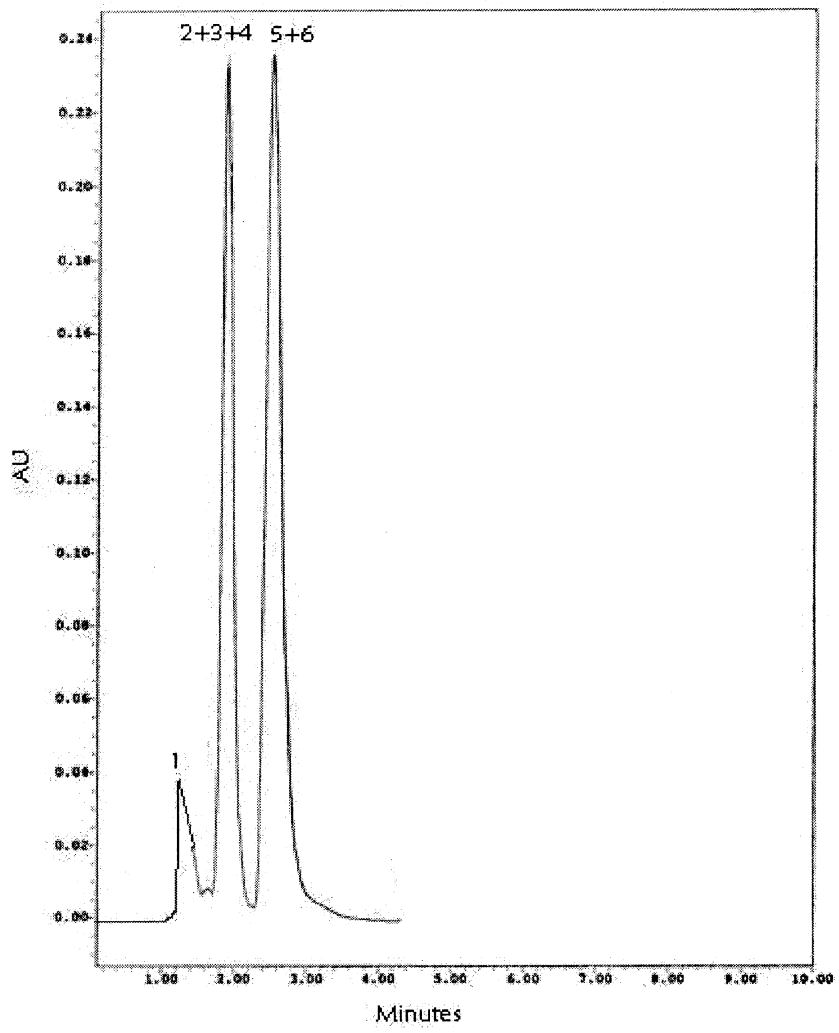


Figure 35. Typical HPLC separation profile for the steroid mixture on the 9-vinylanthracene (fr.rad) column. The mixture was separated isocratically using 100% 100% acetonitrile at a flow rate of 2ml/min and detected at 240 nm. Component identification: 1, estradiol; 2, 11-α acetoxy progesterone ; 3, adrenosterone ; 4, estrone ; 5, corticosterone ; 6, prednisone.

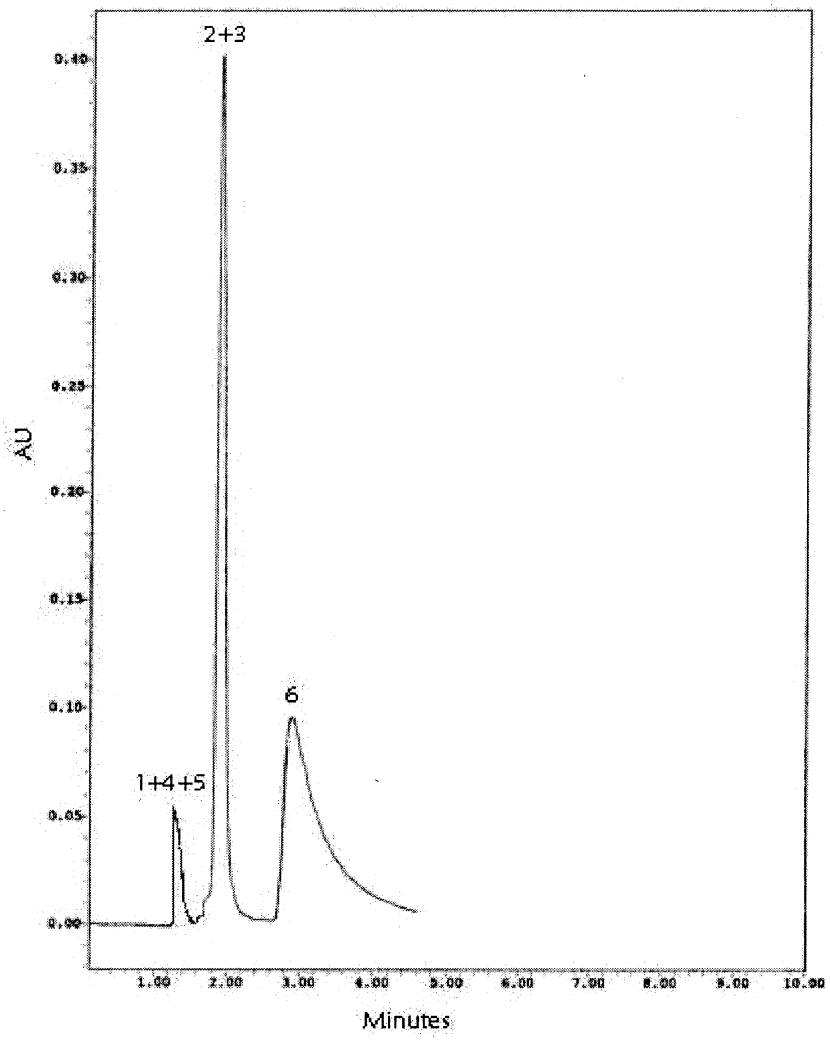


Figure 36. Typical HPLC separation profile for the steroid mixture on the ethisterone (Pt) column. The component identification and separation conditions are the same as in Figure 35.

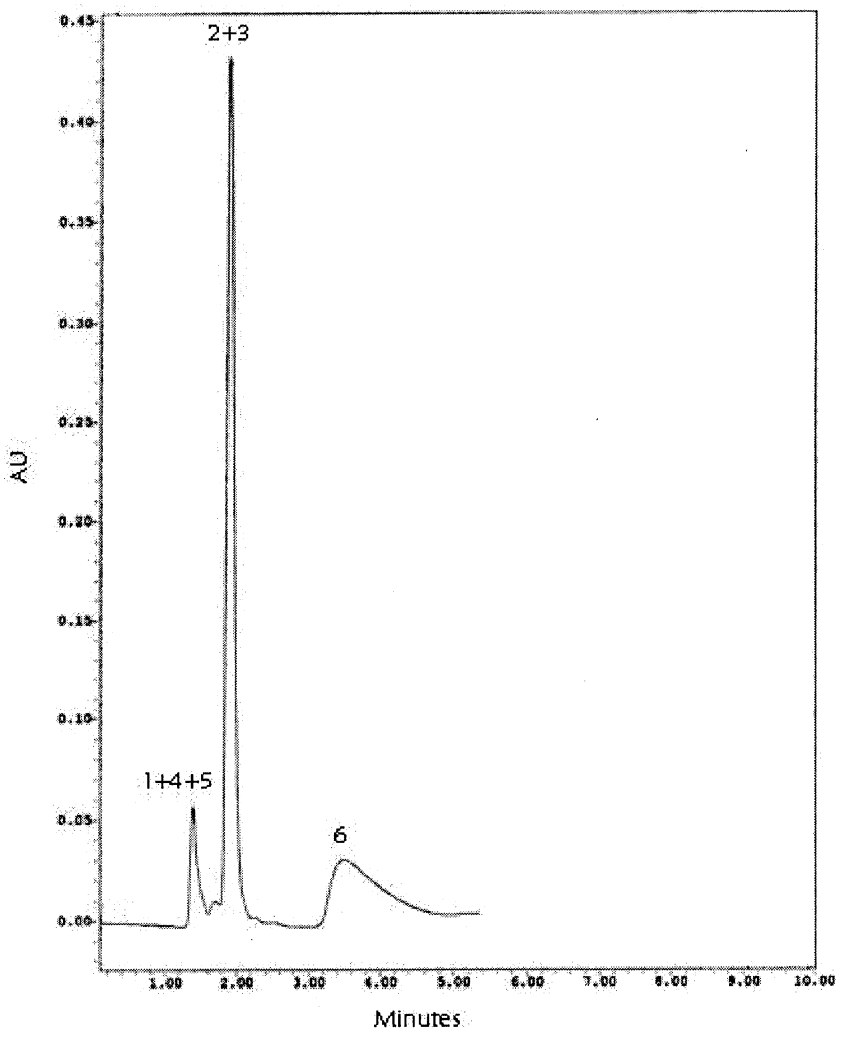


Figure 37. Typical HPLC separation profile for the steroid mixture on the ethisterone (fr.rad) column. The component identification and separation conditions are the same as in Figure 35.

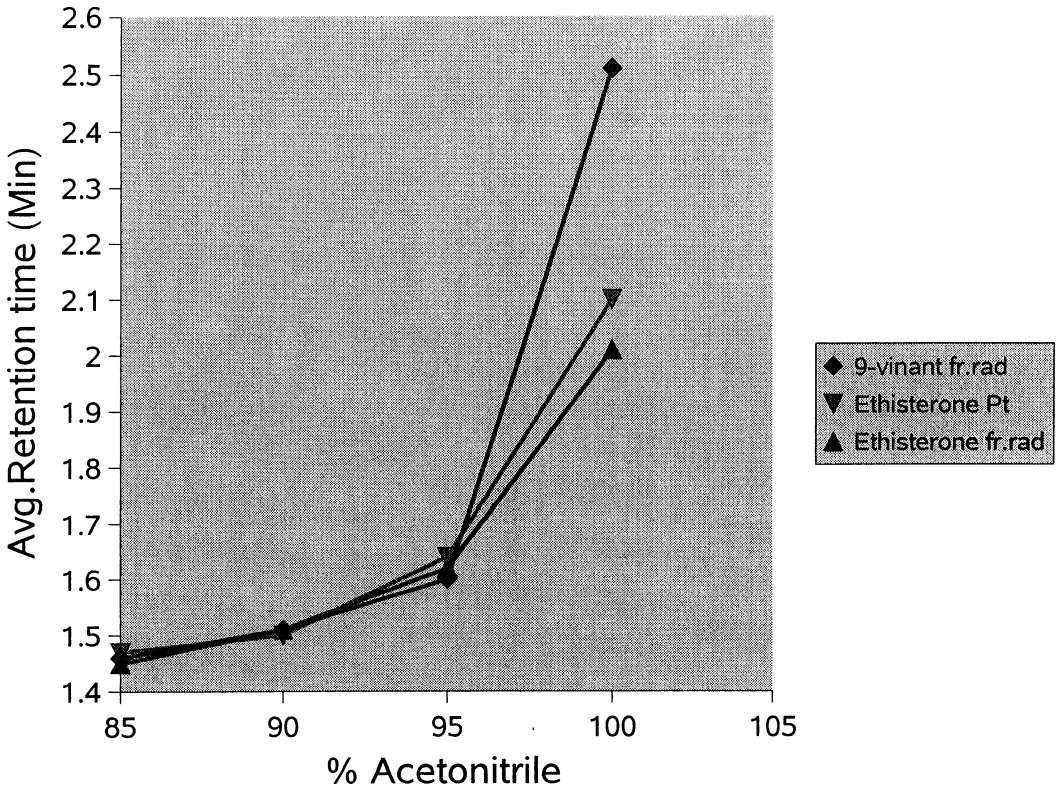


Figure 38. Comparison of the retention behavior of the 3 columns using corticosterone as the reference in the reversed- phase.

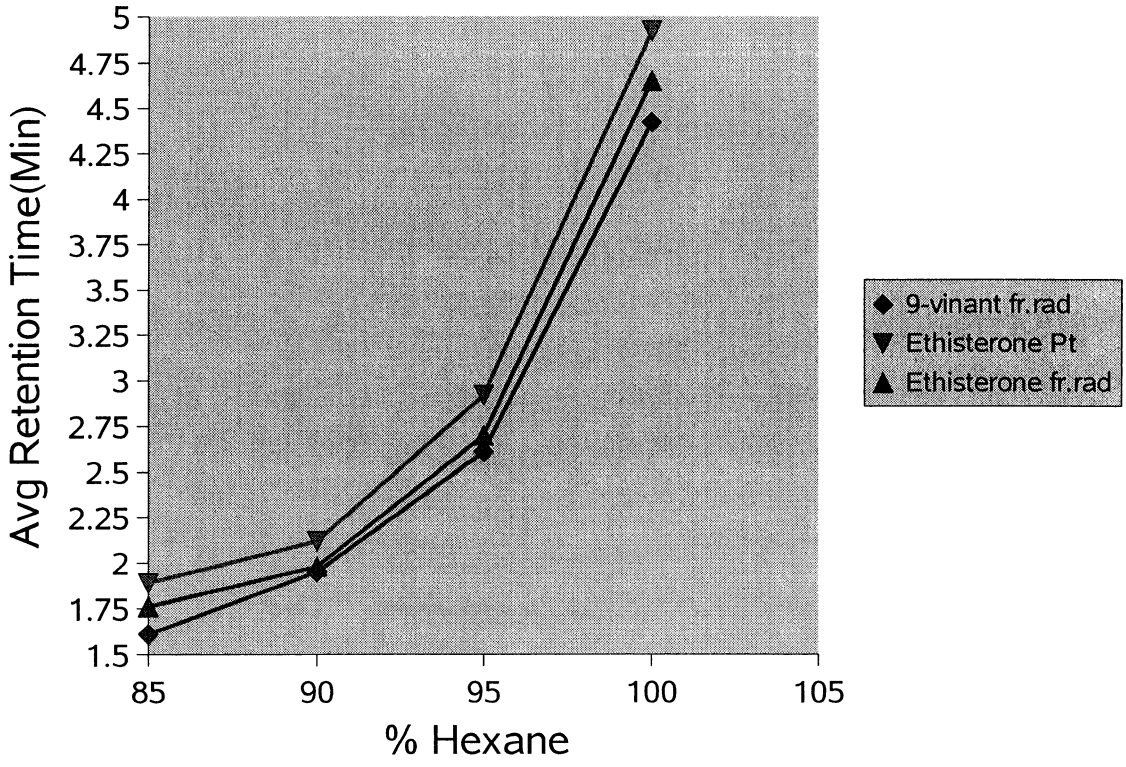


Figure 39. Comparison of the retention behavior of the 3 columns using corticosterone as the reference in the normal phase.

4. CONCLUSIONS

The primary goals of this study, i.e., synthesis of bonded phases using the silanization/hydrosilation method, have been met. The hydrosilation method of bonding an organic moiety to the hydride surface was successful using both Pt and free radical catalysts. Results from DRIFT and ^{13}C CP-MAS NMR spectrum provided useful and conclusive confirmation for the success of bonding. The failure of 9,10-bis (phenylethynyl)anthracene bonded phase in performing chromatographic separations can be explained by the probable reduction of the Pt catalyst when exposed to polar mobile phase solvents. The amount of carbon loaded on this bonded phase which was determined by elemental analysis was also very poor. The carbon percentage on the other 3 columns 9-vinylanthracene (fr.rad), ethisterone (Pt) and ethisterone (fr.rad) was also low owing to their high molecular weights. This had a significant effect on their chromatographic behavior. The three columns had no significant separation of tocopherols and PAHs when operating under reversed-phase conditions. However, all the columns separated the steroid mixture partially in the aqueous normal phase using 100% acetonitrile as the mobile phase. In the normal phase mode, tocopherols and PAHs were separated on all the columns using 100% hexane as the mobile phase. With all the selected test solutes, a trend of a decrease in the retention times with increased mobile phase polarities was observed on all the columns which is consistent with normal phase behavior.

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